Biofilms, persistence, and antibiotic resistance

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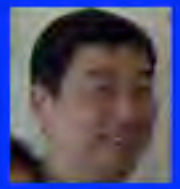
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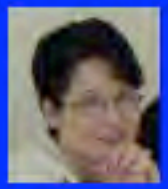
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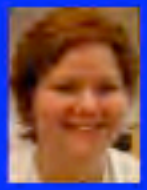
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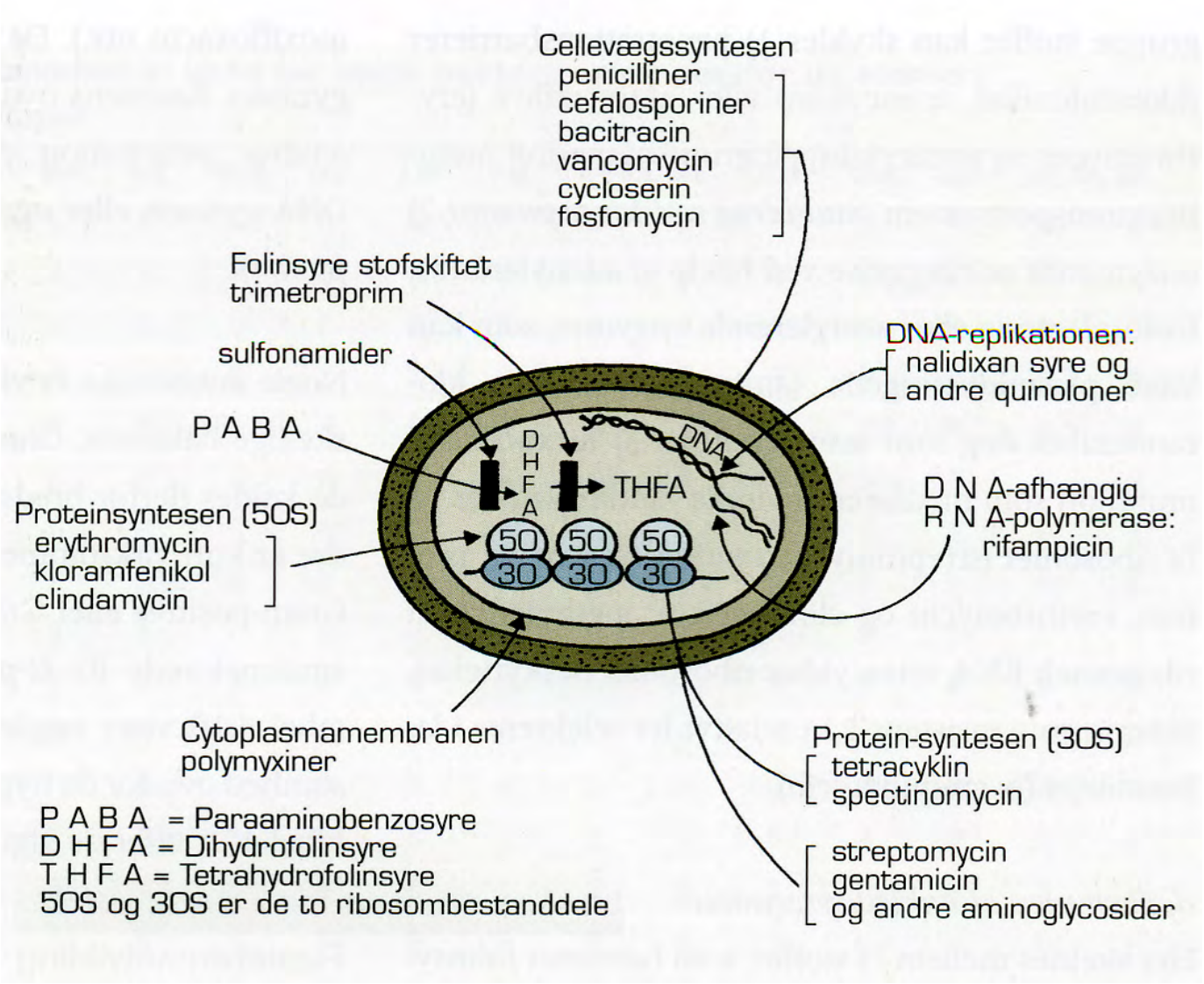
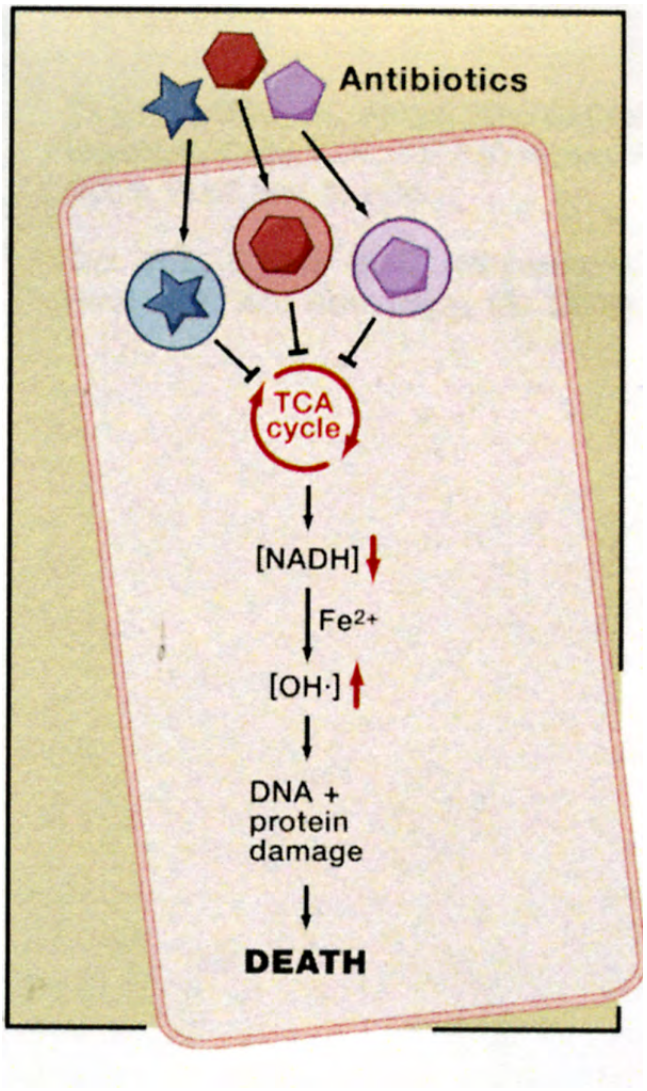
TARGETS FOR ANTIBIOTICS



Common mechanism for cell death induced by bactericidal antibiotics (beta-lactams, aminoglycosides, quinolones)



Antibiotics binds to their targets and this also interfere with the general metabolisme including the TCA cyclus, leading to reduced production of NADH and therefore increase of oxygen radicals (superoxide, peroxide) which react with Fe^{2+}->Fe^{3+} leading to $OH^{\cdot}$ (hydroxylradical) formation and subsequent death of the bacterial cells due to damage on DNA, protein and lipids. (Wright. Cell 130:781; 2007. Kohanski. Cell 130:797; 2007)

Anwar & Costerton: Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of P. aeruginosa. AAC 1990;34:1666-71

FIG. 2. Kinetics of killing of planktonic cells of mucoid (UAM 12 or 492) or nonmucoid (ATCC 27835) *P. aeruginosa* strains by a combination of piperacillin and tobramycin. Symbols: ●, 200 μg of piperacillin plus 5 μg of

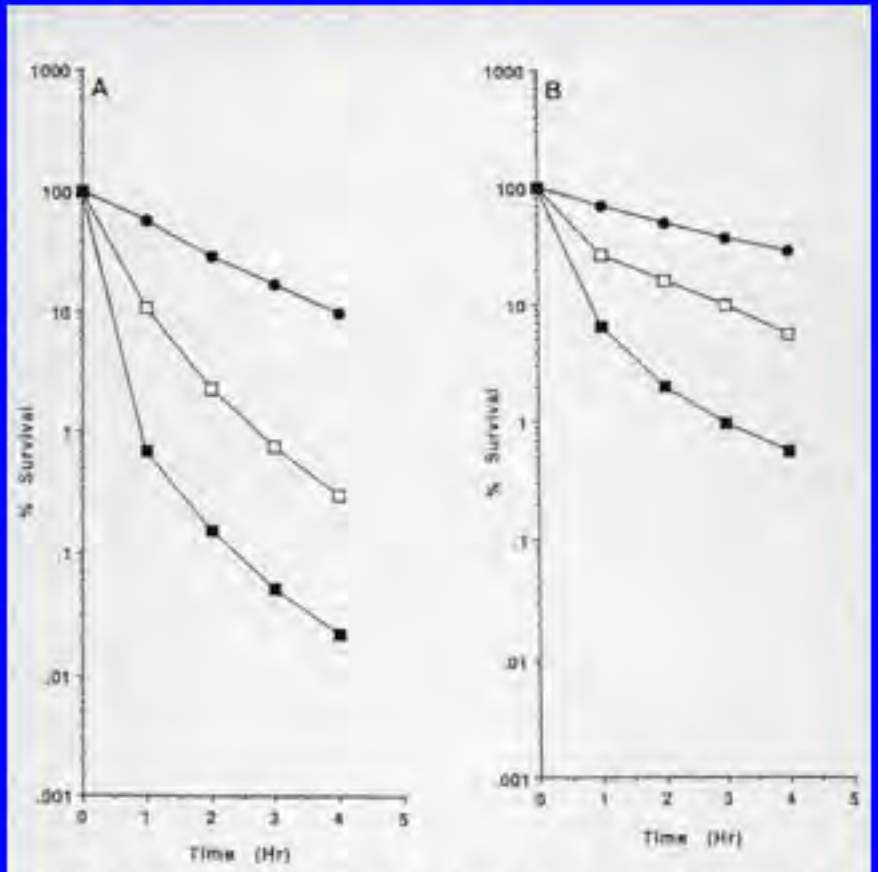
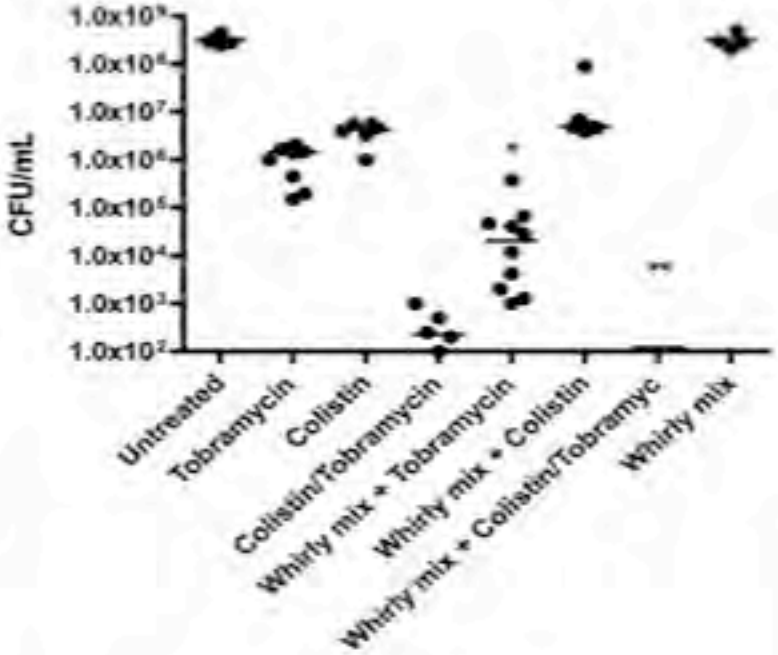


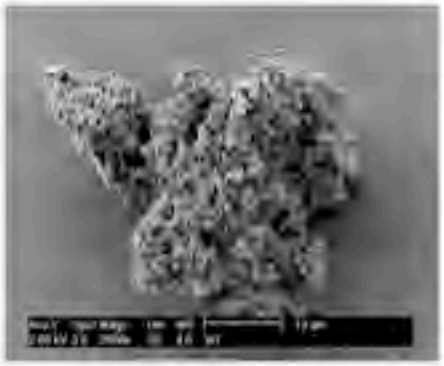
FIG. 6. Kinetics of killing of old sessile bacteria of nonmucoid *P. aeruginosa* ATCC 27835 (A) and mucoid *P. aeruginosa* (UAM 12 or 492) (B) by a combination of piperacillin and tobramycin. Symbols: ●, 200 μg of piperacillin plus 25 μg of tobramycin per ml; □, 200 μg of piperacillin plus 50 μg of tobramycin per ml; ■, 200 μg of

Planktonic P. aeruginosa killed by tobramycin (T) and piperacillin (P)

Old biofilm P. aeruginosa, left Non-mucoid, right: Mucoid. Cannot be killed by T + P since MIC increases 1000 fold in a biofilm

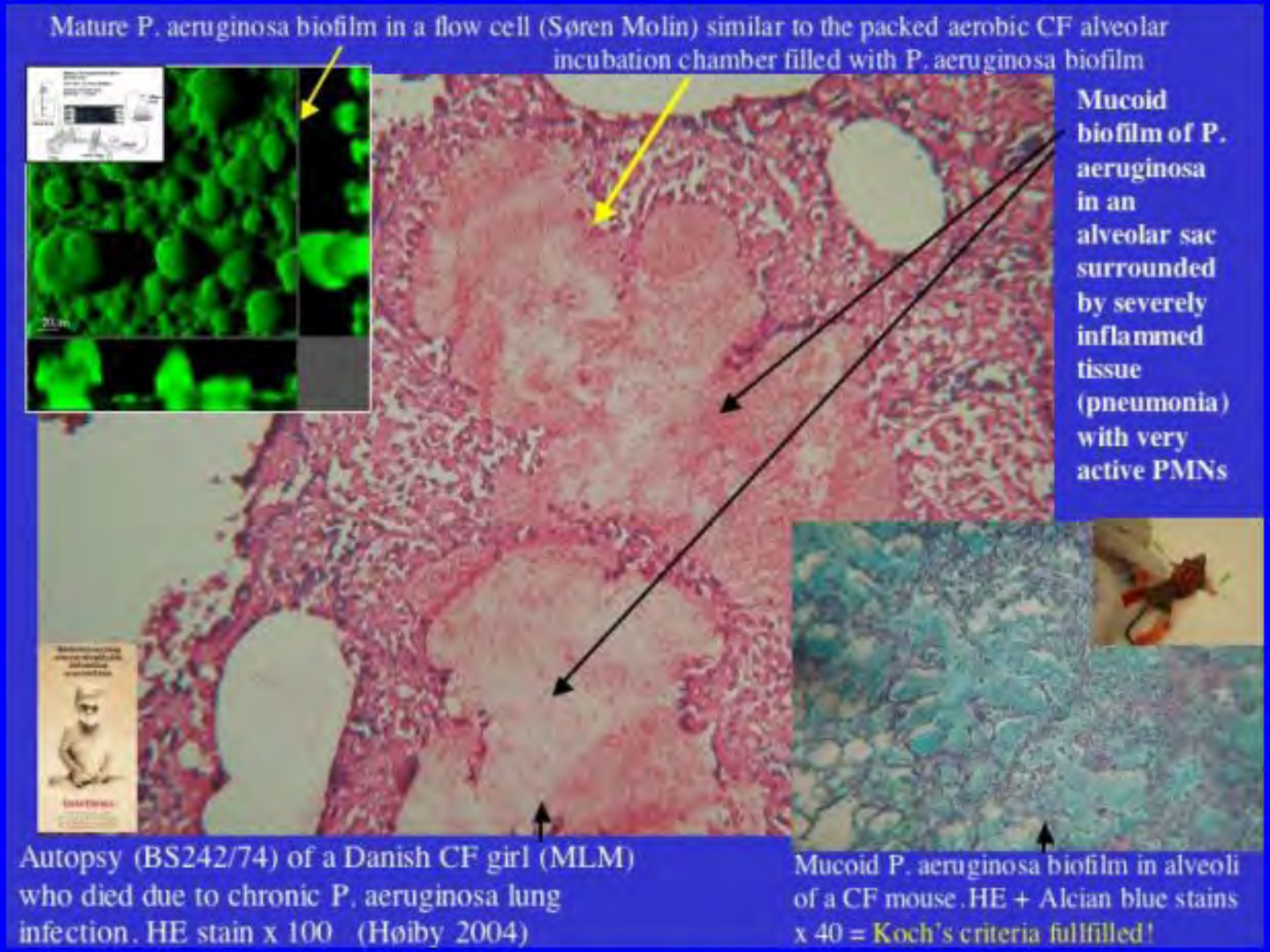


(**100µg/ml Tobramycin** and **25µg/ml Colistin** were applied for 24h)



Aggregates are tolerant (resistant)
Synergistical effect of antibiotics
Disruption of aggregates lowers tolerance (susceptible)

(Alhede, M. Kragh, K.N., , M., Qvortrup, K., Allesen-Holm, M., van Gennip, M.., Christensen, L.D., Jensen, P.Ø., Nielsen, A.K., Parsek, M., Wozniak, D., Molin, S., Tolker-Nielsen, T., Høiby, N., Givskov, M., Bjarnsholt, T.: Phenotype of non-attached Pseudomonas aeruginosa aggregates resemble surface attached biofilm. PLoS ONE. 6 (11):2011.)



(Hoffmann, Rasmussen, Jensen, Stub, Hentzer, Molin, Ciofu, Givskov, Johansen, Høiby: Novel mouse model of chronic Pseudomonas aeruginosa lung infection mimicking cystic fibrosis. Infect. Immun. 73:2504-14; 2005)

CASE

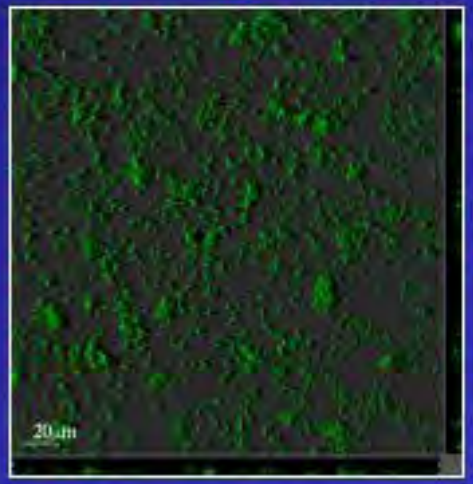
Complex Adaptive Systems Ecology

Biofilm Development

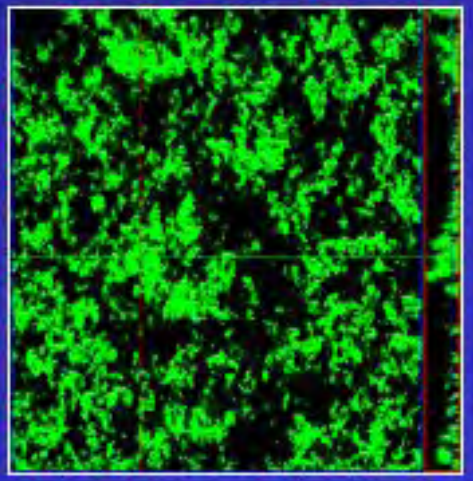
Biofilm development in flow-chambers as seen through the scanning confocal laser microscope

1) Reversible attachment

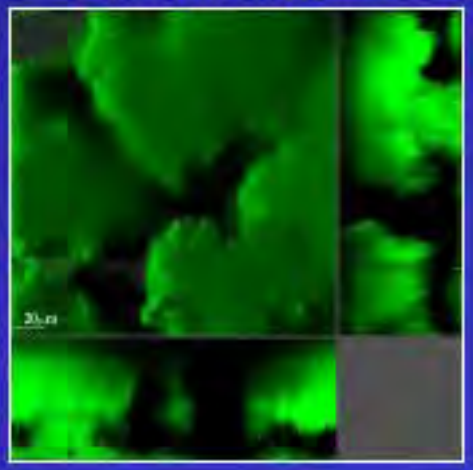
2) Irreversible attachment



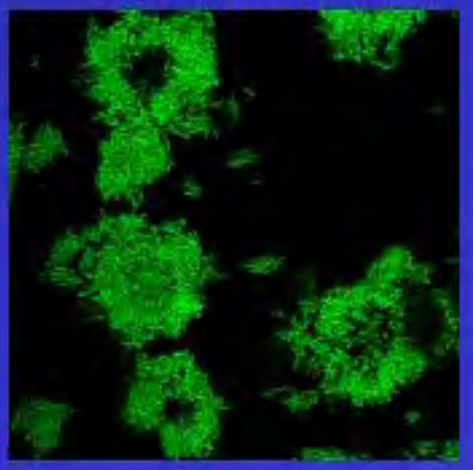
3) Cell proliferation



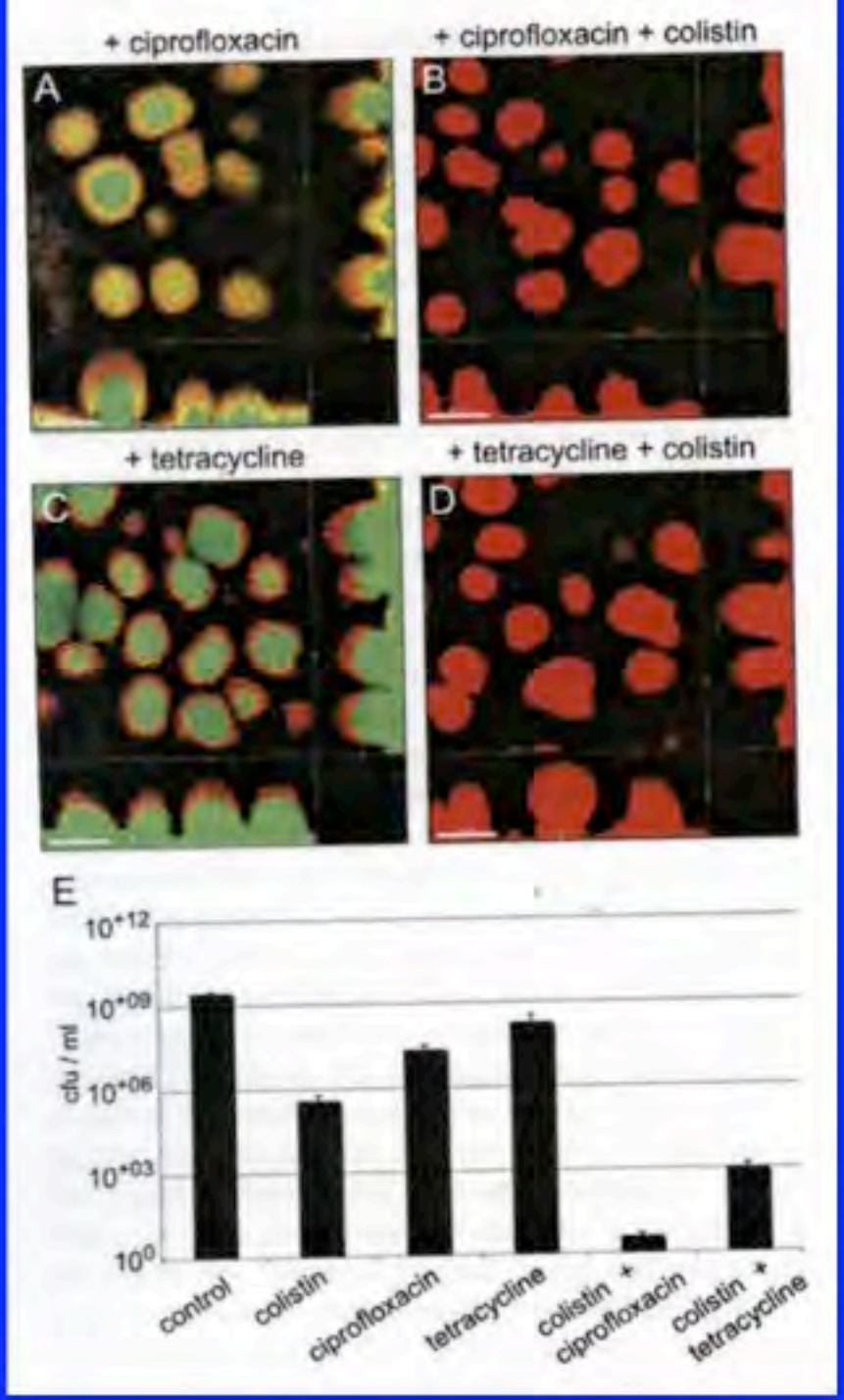
4) Biofilm maturation



5) Dissolution



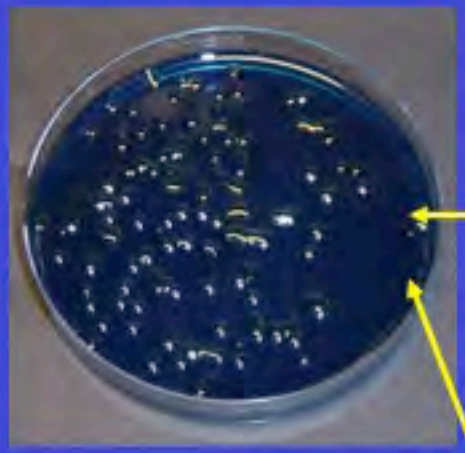
Søren Molin BioCentrum DTU

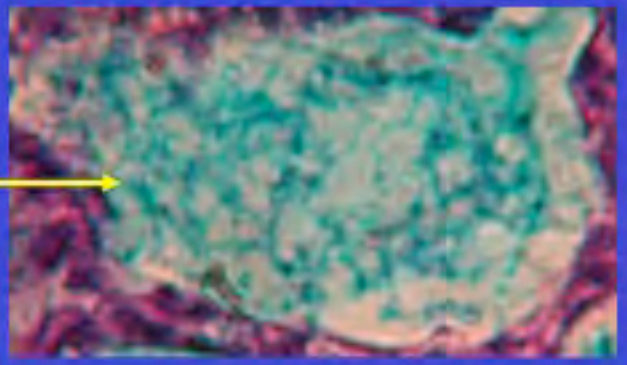


Pamp, Gjermansen, Krogh Johansen, Tolker-Nielsen et al. Tolerance to the antimicrobial peptide colistin in P. aeruginosa biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. Molecular Microbiology 68:223-40; 2008

Synergism – or additive effect – of:
colistin and ciprofloxacin
and of
colistin and tetracycline

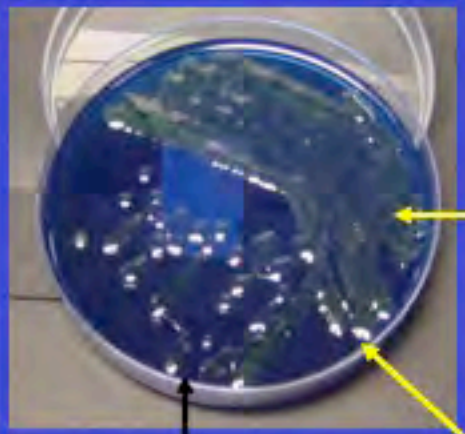
CF MOUSE lung infection with stable mucoid P. aeruginosa 57388A for 7 days, LEFT: Mucoid colonies

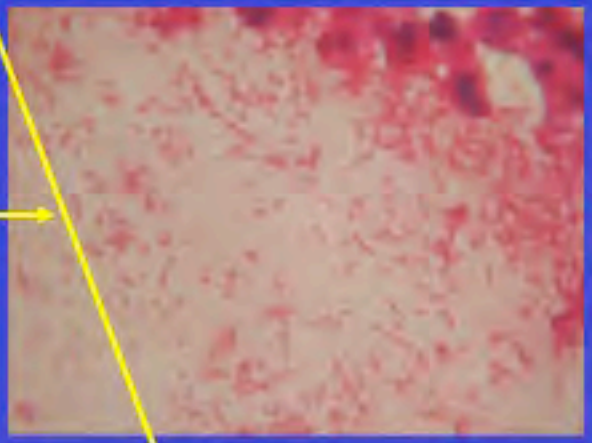




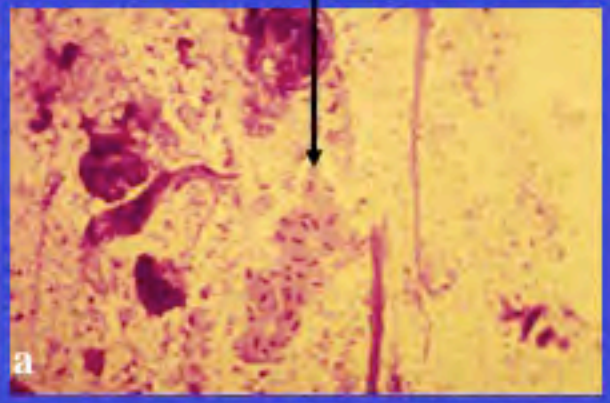
RIGHT: CF MOUSE alveole with mucoid P. aeruginosa biofilm. Staining: Alcian blue for alginate

CF PATIENTS with chronic P. aeruginosa lung infection. LEFT: Mucoid colonies from BAL (CF LFS)

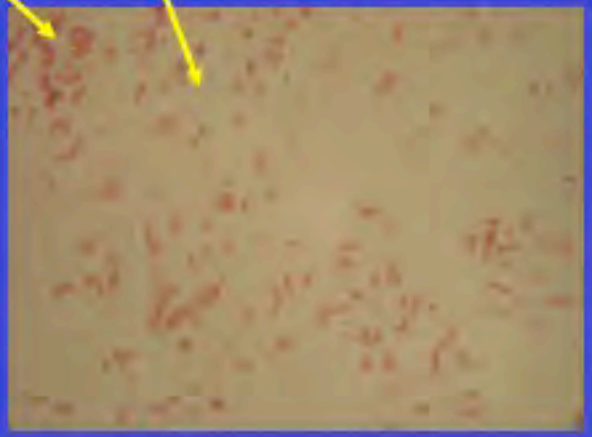




RIGHT: CF PATIENT: Autopsy (CF MLM), alveole with mucoid biofilm. Staining: Gram



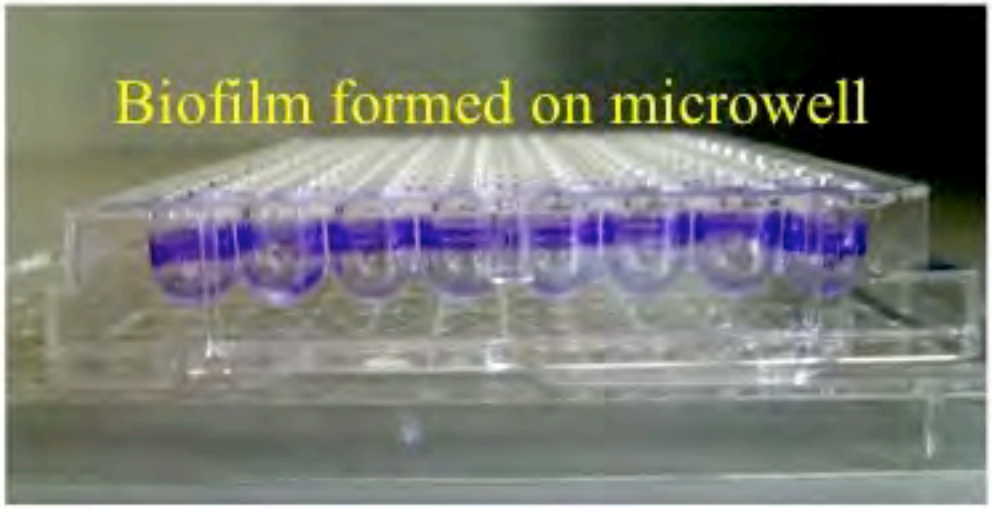
LEFT: Sputum with mucoid biofilm of P. aeruginoa from CF patient

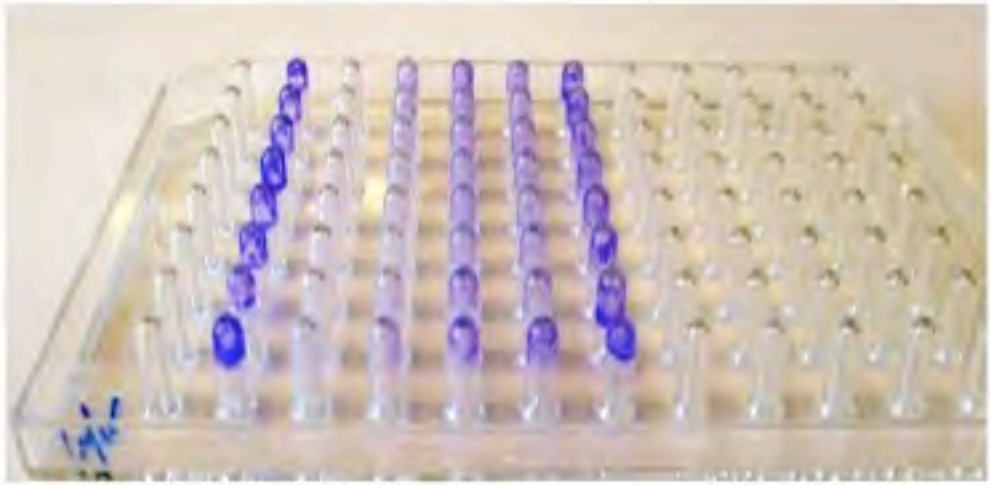


RIGHT: Smear from a mucoid colony of P. aeruginosa Staining: Gram

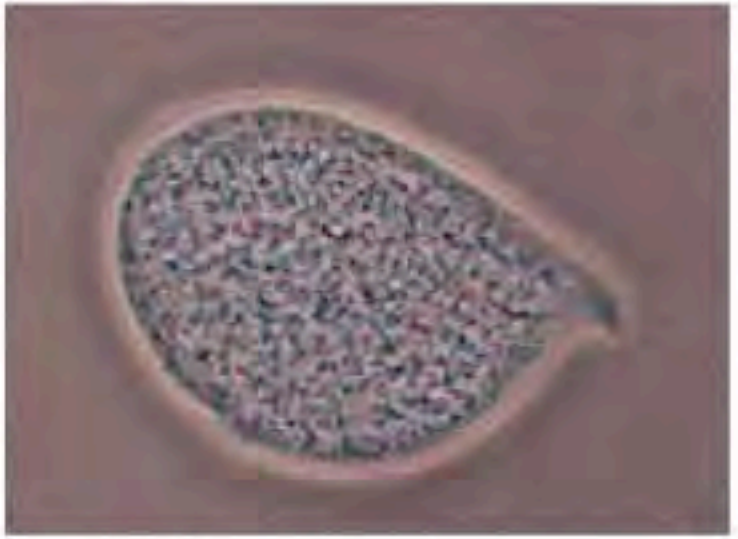
HØIBY 2003

Materials and Methods





Non mucoid PAO1 Biofilm formed on peg-lid of microplate



Alginate beads (Ø50-100μm) for *in vitro* and *in vivo* biofilm model (artificial mucoid of PAO1)

Wang. AAC. 55:4469-4474; 2011

(Wang, Song, Wu & Høiby 2010)

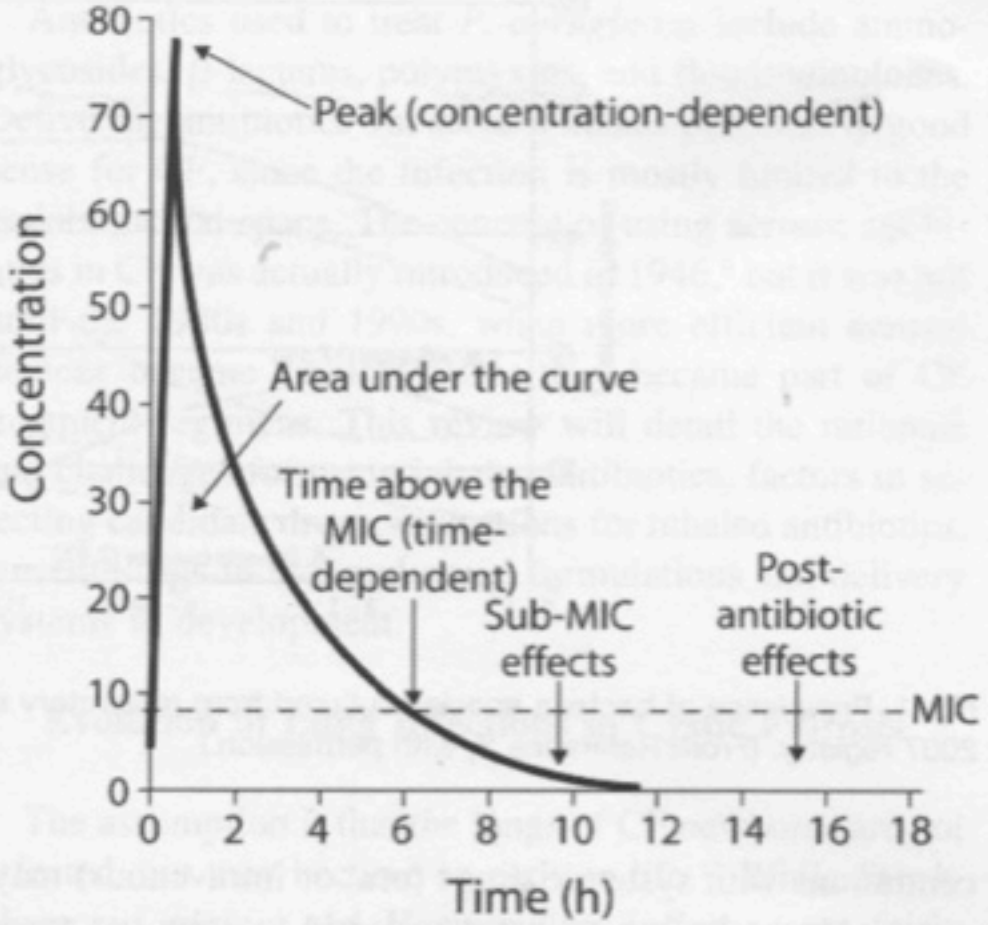
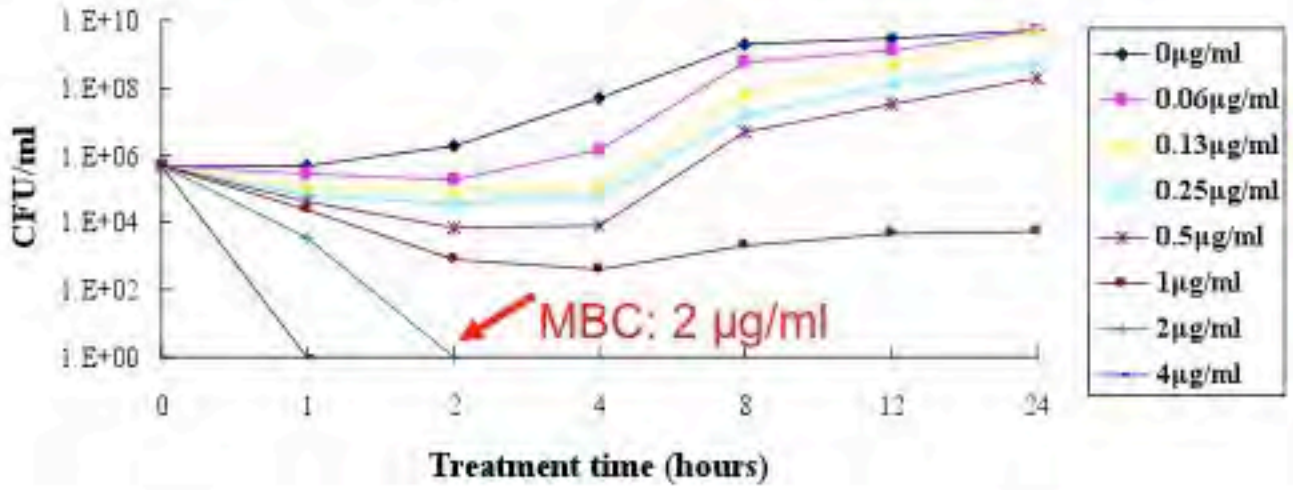


Fig. 3. Antibiotic pharmacodynamics. The graph is a representative concentration-versus-time curve of an antibiotic in the compartment of interest (in this case, the lungs). After inhalation, the level is very high, then falls due to absorption into the bloodstream or elimination through airway clearance. β-lactam antibiotics demonstrate time-dependent killing (the longer the time above the minimum inhibitory concentration [MIC] of the bacteria, the better). Aminoglycosides and fluoroquinolones demonstrate concentration-dependent killing (a high ratio of average maximum serum concentration to MIC or area-under-the-curve [AUC] to MIC work best).

Planktonic non-mucoid P. aeruginosa PAO1

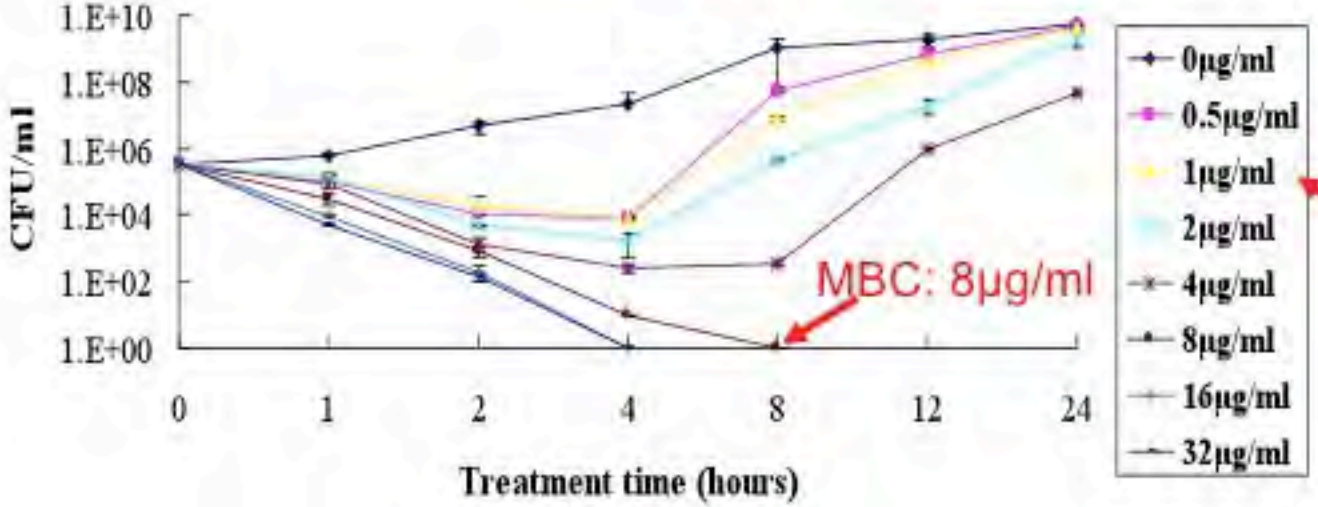
MBC: minimal bactericidal concentration



Ciprofloxacin

Planktonic MIC:
0.06 µg /ml (E-test)

Concentration-dependent killing



Imipenem

Planktonic MIC:
1 µg /ml (E-test)

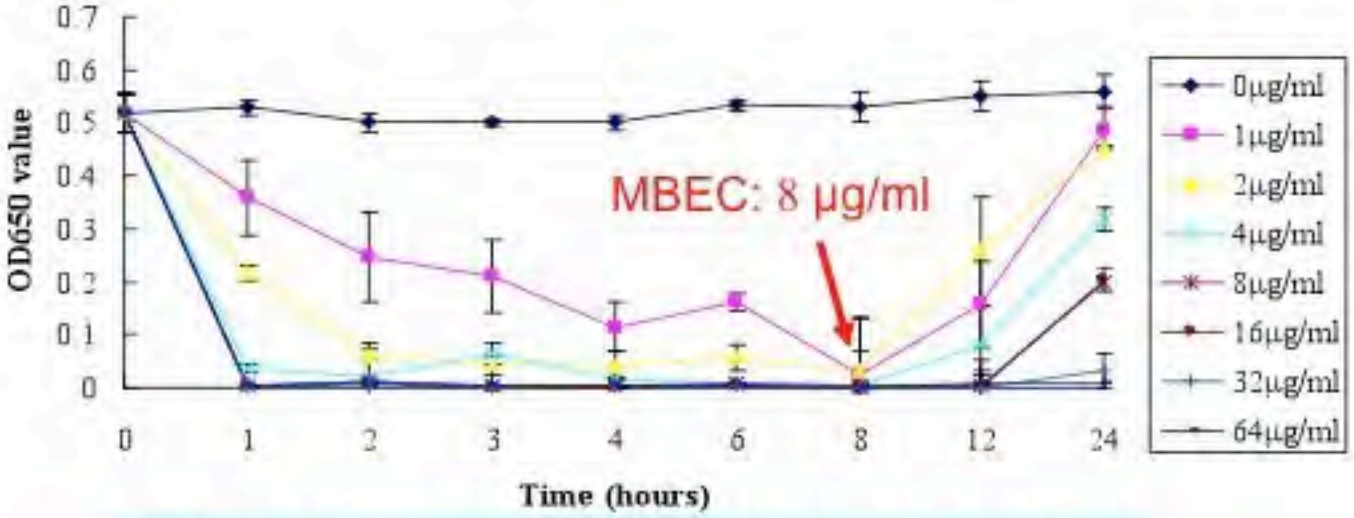
Time-dependent killing

Wang. AAC. 55:4469-4474; 2011.

(Wang, Song, Wu & Høiby 2010)

Day 1 biofilm of non-mucoid PAO1

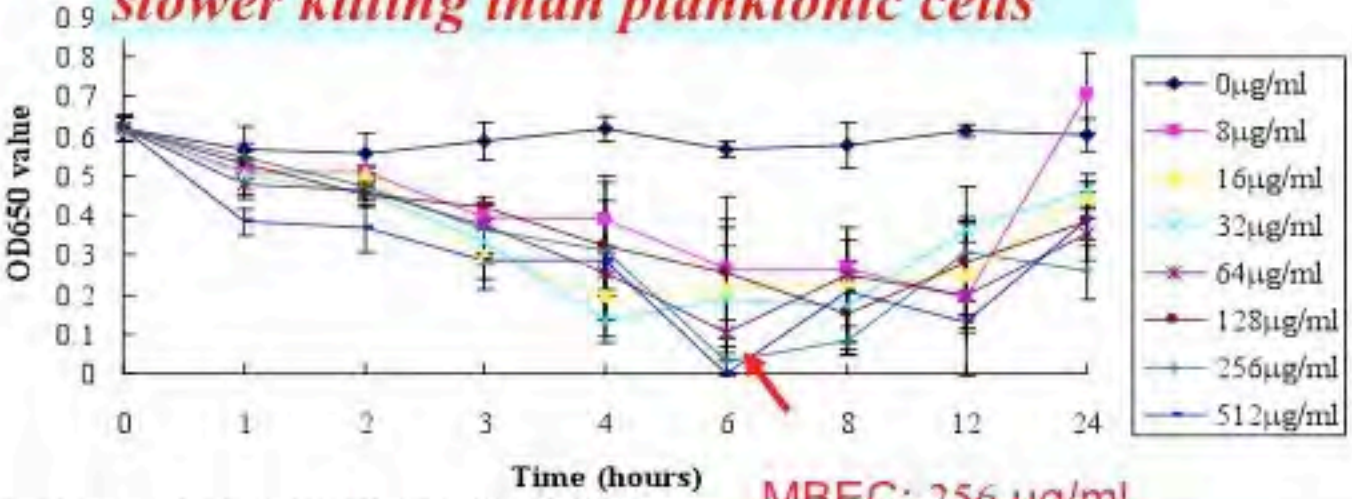
MBEC: minimal biofilm eradication concentration



Ciprofloxacin

Concentration-dependent killing

Higher concentration required and slower killing than planktonic cells



Imipenem

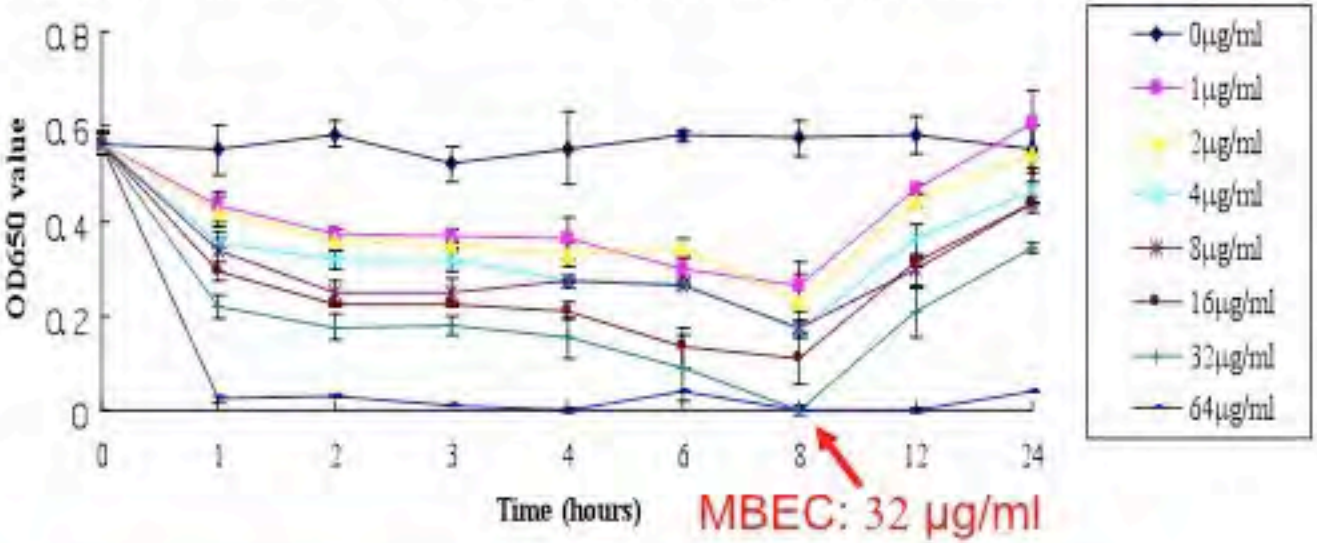
Time-dependent killing

Wang et al. AAC 55:4469-4474; 2011

(Wang, Song, Wu & Høiby 2010)

Day 3 biofilm of non-mucoid PAO1

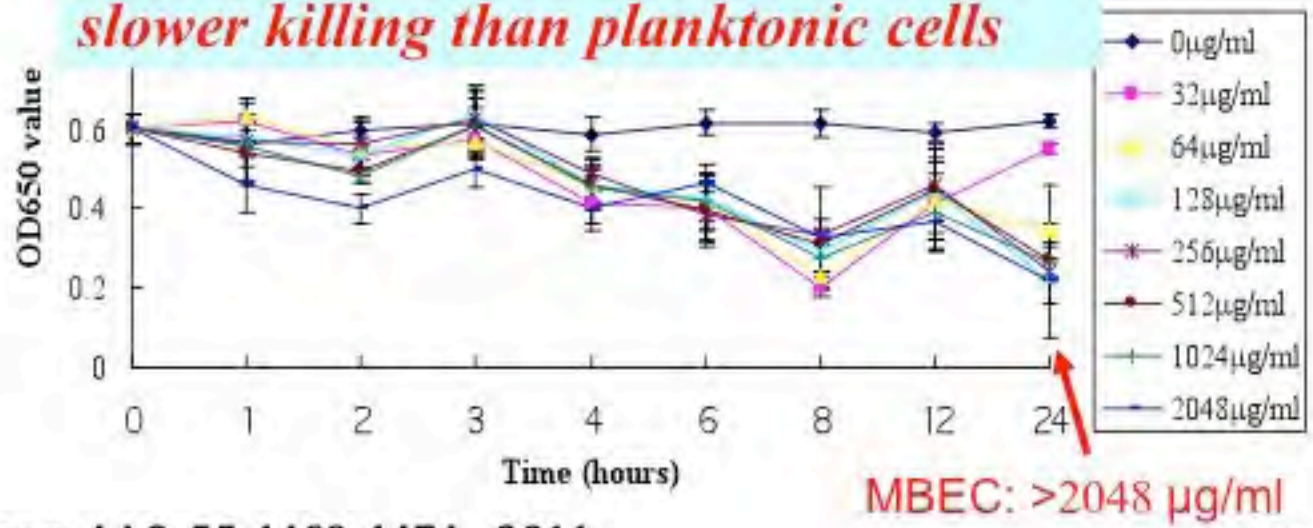
MBEC: minimal biofilm eradication concentration



Ciprofloxacin

Concentration-dependent killing

Higher concentration required and slower killing than planktonic cells



Imipenem

Time-dependent killing

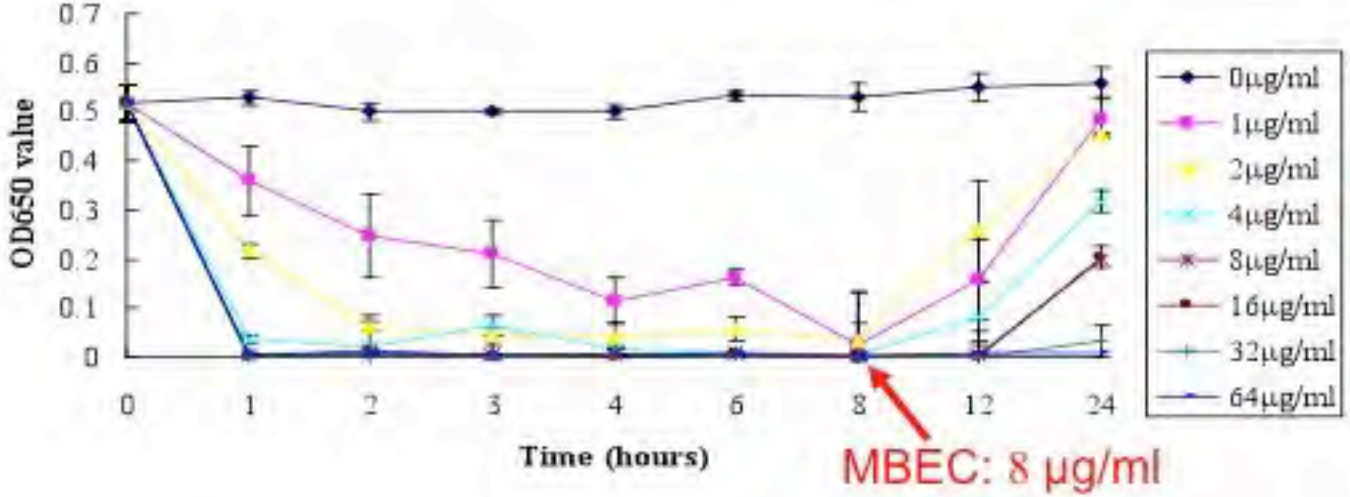
Wang. AAC. 55:4469-4474; 2011

(Wang, Song, Wu & Høiby 2010)

Non-mucoid PAO1 vs. mucoid PDO300 of Day 1 biofilm

(PAO1 Alg +)

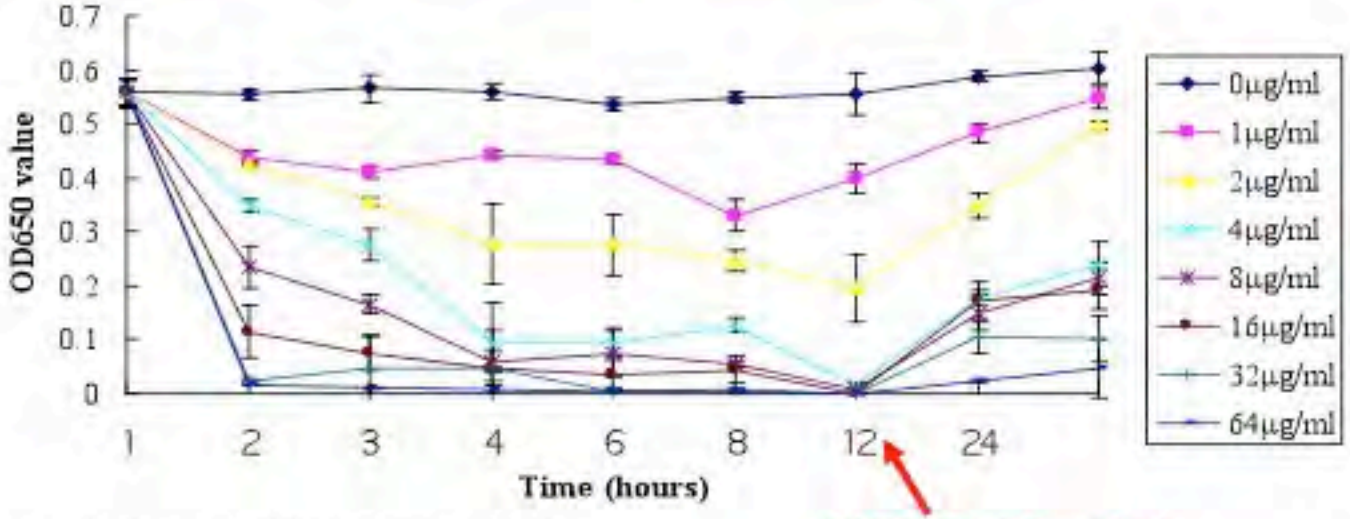
MBEC: minimal biofilm eradication concentration



Ciprofloxacin

Non-mucoid PAO1

Day 1 biofilm



Ciprofloxacin

Mucoid PDO300

Day 1 biofilm

(Wang, Song, Wu & Høiby 2010)

Wang. AAC. 55:4469-4474; 2011

Summary

Minimal bactericidal concentration (MBC)
minimal biofilm eradication concentration (MBEC)
Minimal biofilm inhibition concentration (MBIC)

	MBC (planktonic) & MBEC (biofilm) (μg/ml)	
	Ciprofloxacin	**Imipenem**
Planktonic	**2**	**8**
Day 1 biofilm (non-mucoid PAO1)	**8**	**256**
Day 1 biofilm (Mucoid PDO300)	**32**	**>256**
Day 3 biofilm	**32**	**>2048**
Alginate beads	**64**	**>256**
PK/PD index	***f*C_{max}/MIC (planktonic)** ***f*AUC/MIC (planktonic)**	***f*T>MIC (planktonic)**
Wang. AAC. 55:4469-4474; 2011	***f*C_{max}/MBIC (biofilm)** ***f*AUC/MIC (biofilm)**	***f*T>MBIC (biofilm)**

(Wang, Song, Wu & Høiby 2010)

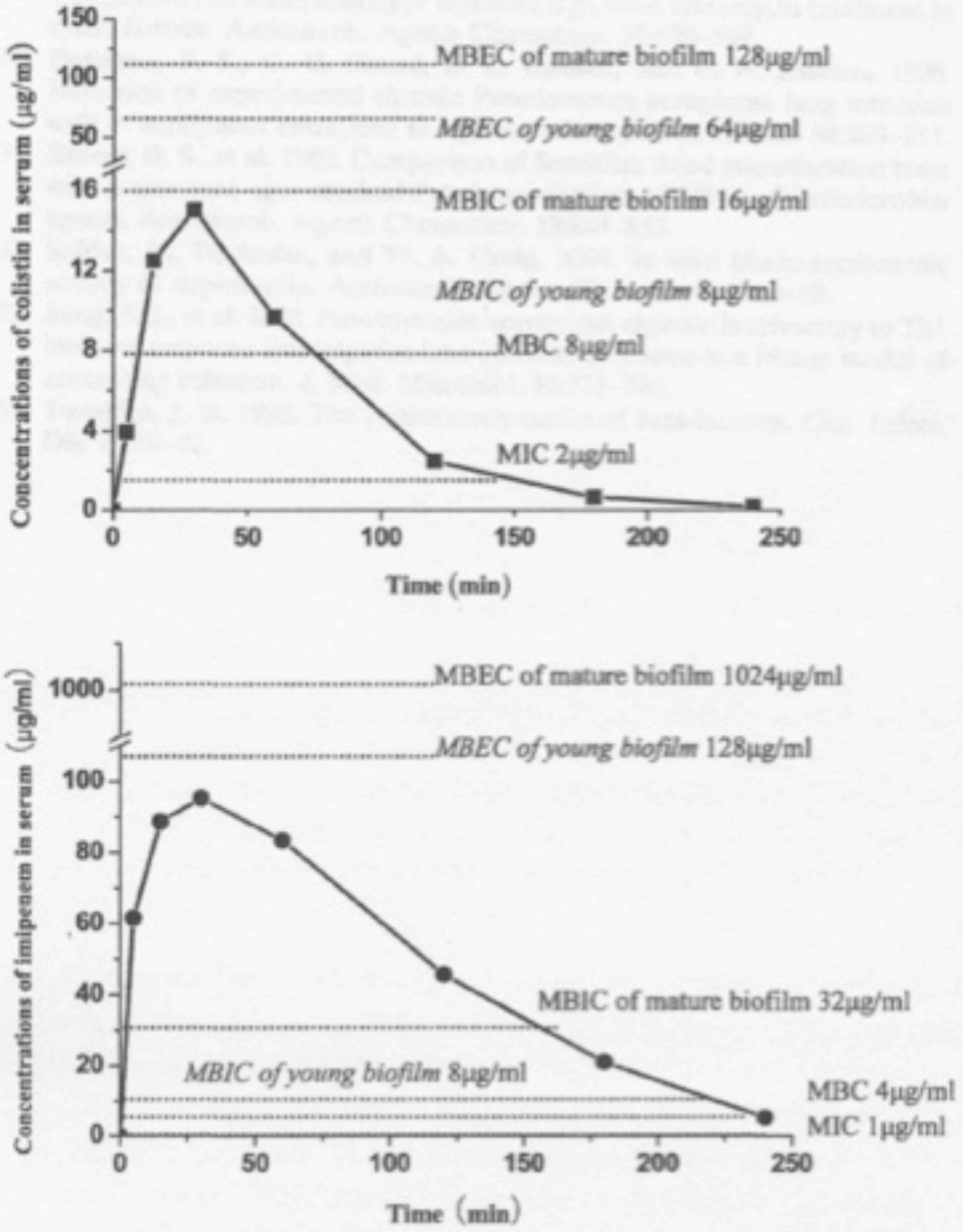
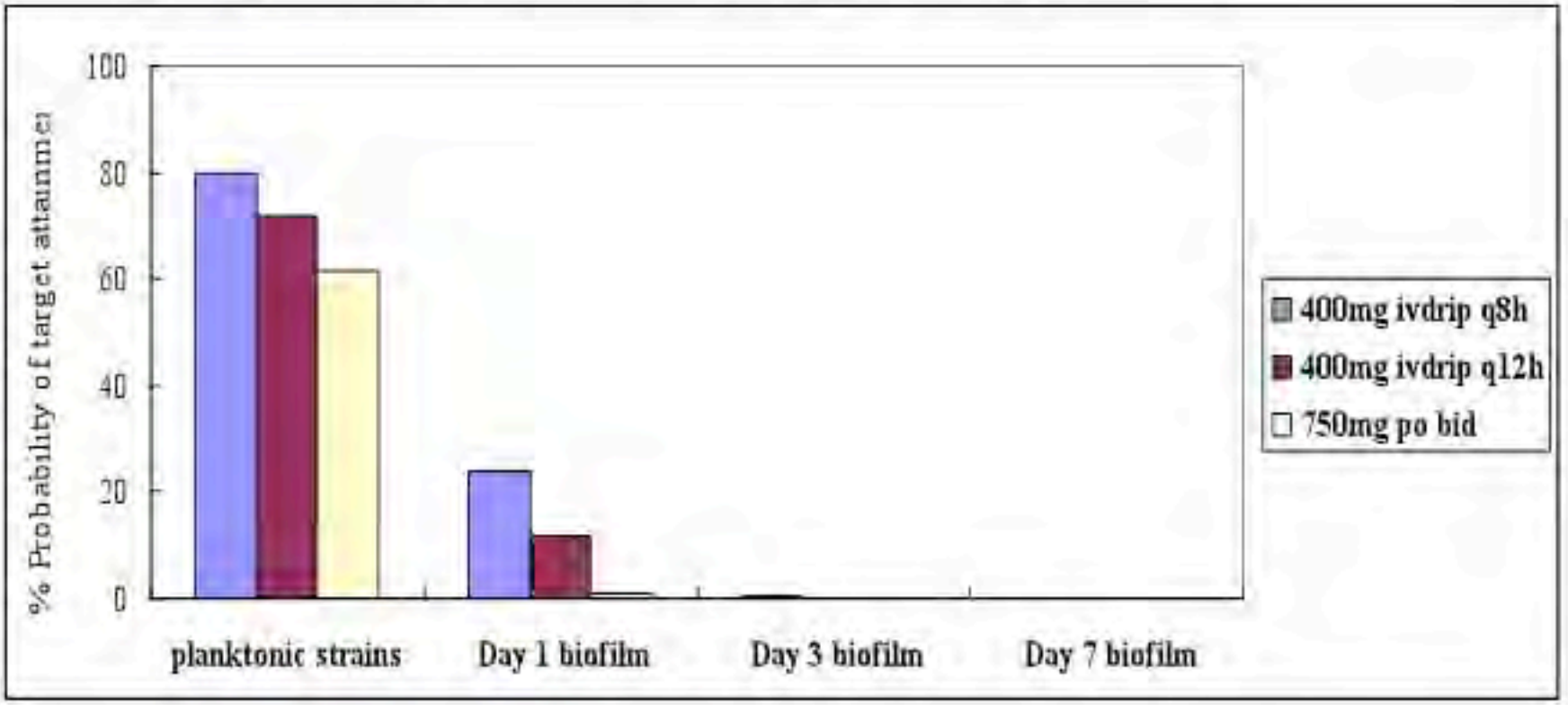


FIG. 4. Pharmacokinetics in mouse serum of colistin and imipenem versus MIC, MBC, MBIC, and MBEC of *P. aeruginosa* PAO165307. ■, 16 mg/kg of colistin; ●, 64 mg/kg of imipenem with one-dose intraperitoneal administration.

Wang. AAC. 55:4469-4474; 2011

Ciprofloxacin Population Pharmacokinetics / Monte Carlo Simulation on CF patients on the basis of published data

※Target: *f*AUC0-24/MIC ratio ≥ 125 (mg · h / liter)



(Alan Forrest, AAC, 1993; Pedersen S. S, JAC, 1987; Montgomery M. J, AAC, 2001)

Wang. AAC. 55:4469-4474; 2011

(Wang, Song, Wu & Høiby 2010)

Biofilm (B) – Planktonic (P) antimicrobial resistance mechanisms

- Stationary phase physiology, low oxygen – slow growth (B)
- Mutations – hypermutators (B, P)
- Beta-lactamase, penetration barrier, alginate, binding to matrix (B, P)
- Tolerance, adaptive resistance, efflux pumps (B, P)
- Persisters (B, P)
- High cell density – Quorum sensing (B, P)

(Høiby, N., Bjarnsholt,T., Givskov, M., Molin, S., Ciofu, O.: Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents 35:322-32; 2010)

Yang, Haagensen, Jelsbak, Johansen, Sternberg, Høiby, Molin. In situ growth rates and biofilm development of P. Aeruginosa populations in chronic lung infections. J. Bact. 190:2767-76; 2008

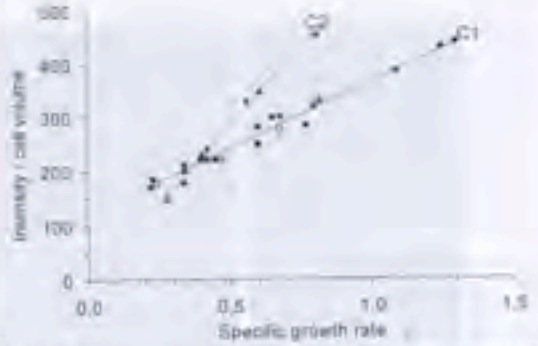


FIG. 3. Cellular content of ribosomes (fluorescence signal intensity per cell volume) inferred by whole-cell hybridization with a fluorescence-labeled 16S rRNA probe of balanced cultures grown in defined media supporting different specific growth rates. The strains and culture conditions used are described in Materials and Methods. The solid line labeled C1 and the dashed line labeled C2 are regression lines. Bacterial isolates of the b genotype (triangles) display the C2 correlation between ribosome content and specific growth rate (r^2 = 0.995). All other isolates tested (circles) display the C1 correlation (r^2 = 0.978). Key data points are highlighted by open symbols: anaerobic growth of isolate p7 sIBJ05 of the b genotype is shown by the open triangle, and open circles show growth of the mucoid isolate p11 s3A1J05 of the 4 genotype in minimal medium supplemented with (from left to right) glucose or glucose with Casamino Acids or in LB medium (fastest growth). Each measurement is the average value obtained from the analysis of the fluorescence signal from more than 100 cells. The standard error of the mean was less than 15% for all measurements. Further details related to the data set (strain information, growth media, growth rate, and intensity per cell volume) are provided in the supplemental material.

Yang, Haagensen, Jelsbak, Johansen, Sternberg, Høiby, Molin. In situ growth rates and biofilm development of P. Aeruginosa populations in chronic lung infections. J. Bact. 190:2767-76; 2008

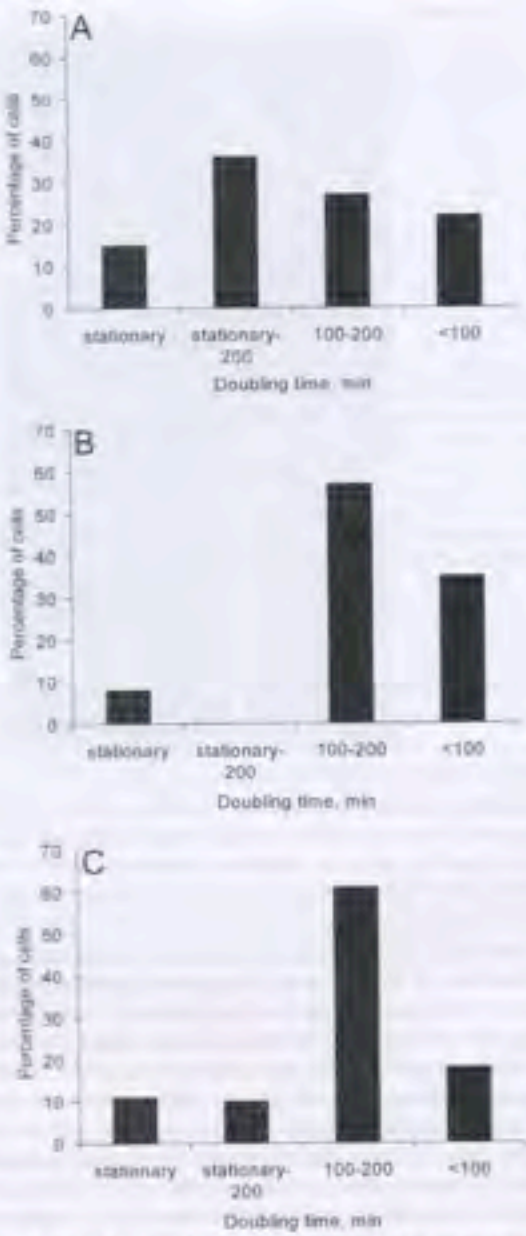


FIG. 5. Distribution of generation times of *P. aeruginosa* cells isolated from sputum samples from CF patients p2 (A), p7 (B), and p11 (C). The cellular ribosome contents (fluorescence signal intensity per cell volume) of bacteria in the samples were measured by whole-cell hybridization using a fluorescently labeled rRNA probe. These measurements were converted into apparent doubling times using the appropriate standard correlations presented in Fig. 3.

- and persisters

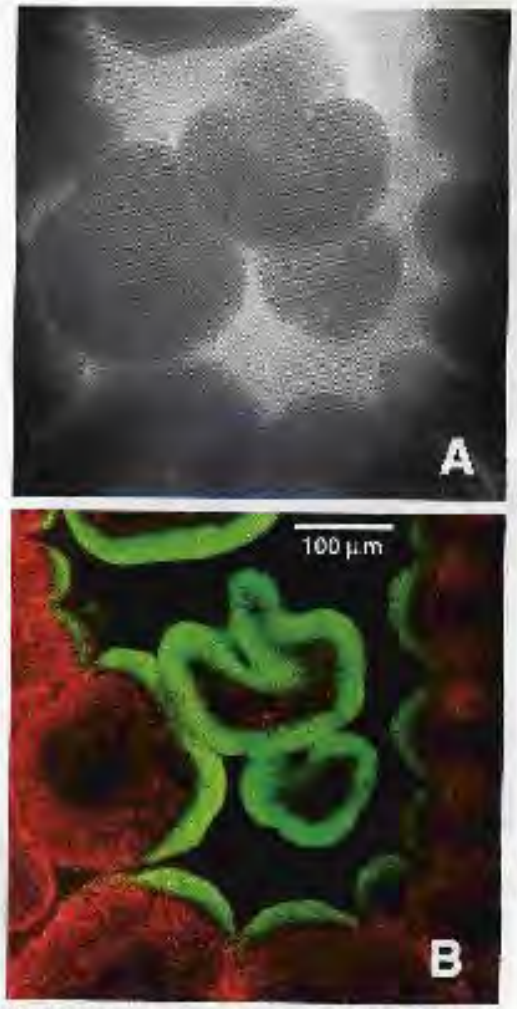


FIG. 8. Stratified pattern of GFP expression in *P. aeruginosa* biofilms grown in glass capillary tubes under continuous flow conditions. Strain PAO1(pADI; was grown for 24 h and then induced with 1 PTG for 4 h. Panel A shows a laser transmission view, and panel B shows a fluorescence image of the same spot. Green areas are due to GFP and red areas are due to the chalcamine B counterstain.

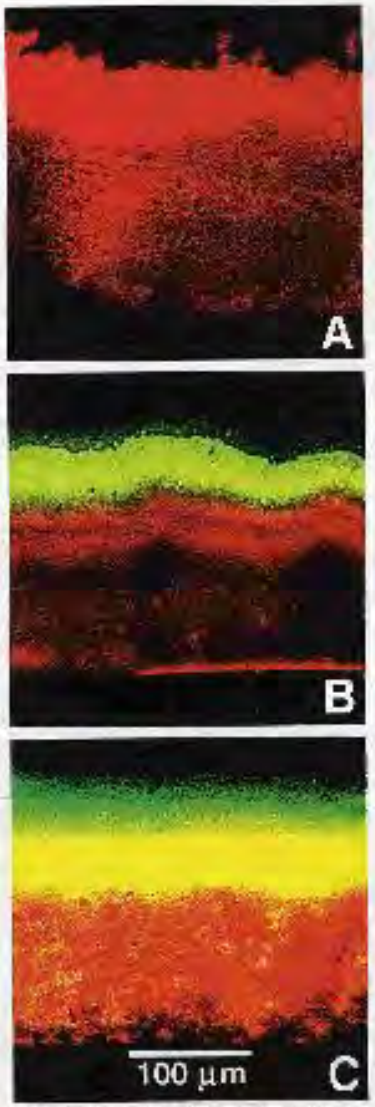


FIG. 4. Stratified patterns of GFP expression in three sections of *P. aeruginosa* colony biofilms. Green areas are due to GFP and red areas are due to the chalcamine B counterstain. Panel A shows a segment excised in which a colony biofilm formed by strain PAO1(pMF230) was not induced. Panel B shows a biofilm of the same strain after 4 h of induction with IPTG. Panel C shows a colony biofilm formed by the reporter strain MF230.

Microbiol. (Werner et al. Stratified growth in P. aeruginosa biofilms. Appl. Environment 70:6188-96; 2004)

Red: control stain of biofilm bacteria

Green = GFP = metabolic active bacteria at the biofilm surface

Biofilm (B) – Planktonic (P) antimicrobial resistance mechanisms

- Stationary phase physiology, low oxygen – slow growth (B)
- Mutations – hypermutators (B, P)
- Beta-lactamase, penetration barrier, alginate, binding to matrix (B, P)
- Tolerance, adaptive resistance, efflux pumps (B, P)
- Persisters (B, P)
- High cell density – Quorum sensing (B, P)

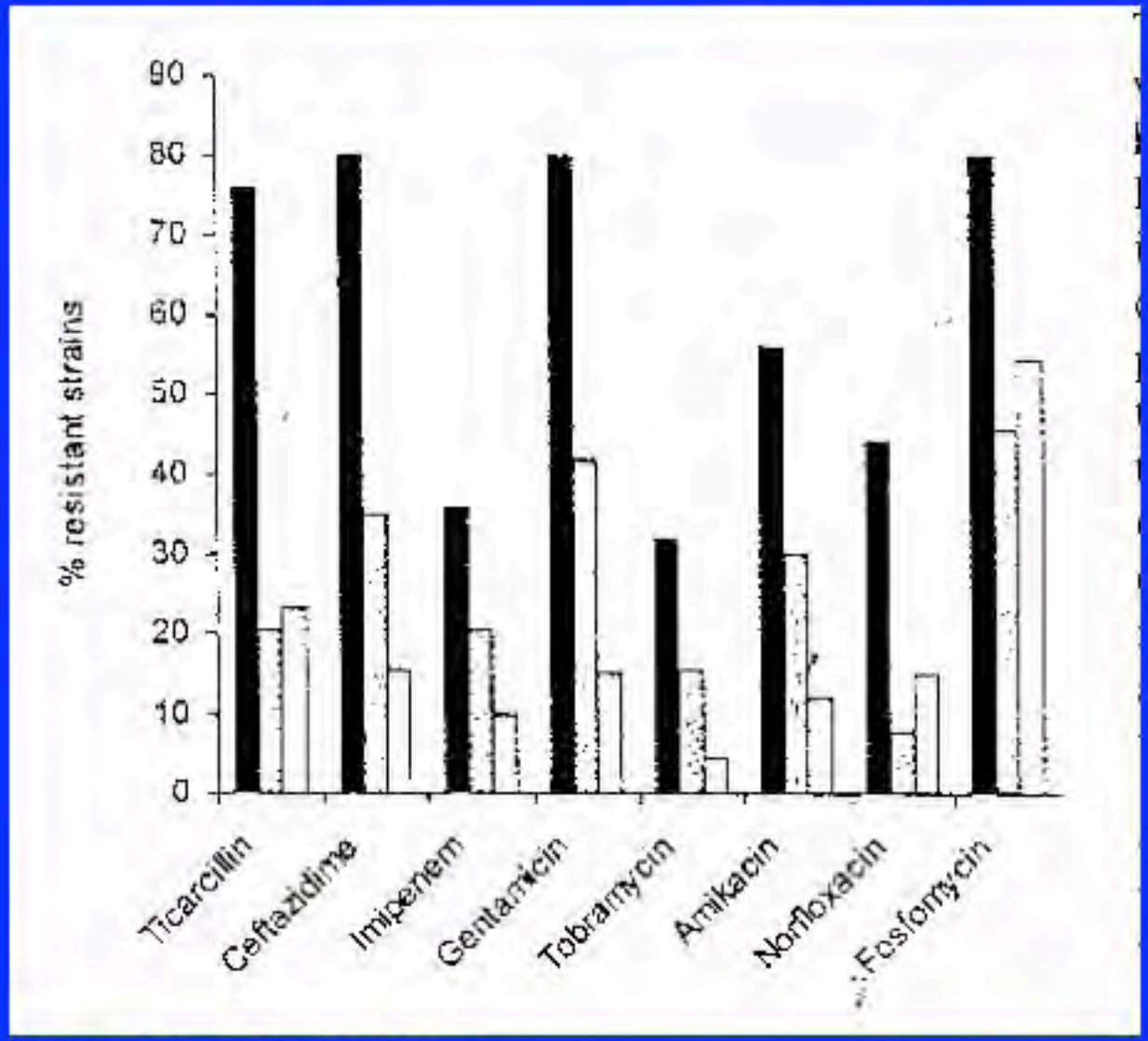
(Høiby, N., Bjarnsholt,T., Givskov, M., Molin, S., Ciofu, O.: Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents 35:322-32; 2010)

Mutation frequencies in biofilm

	Mutation freq. (rifampicin)	8oxodG/10^6dG
PAO1 planktonic	$1xE^{-9}$	22.26
PAO1 biofilm	$2.5xE^{-7}$	57.75

(Mandsberg,L. 2004)

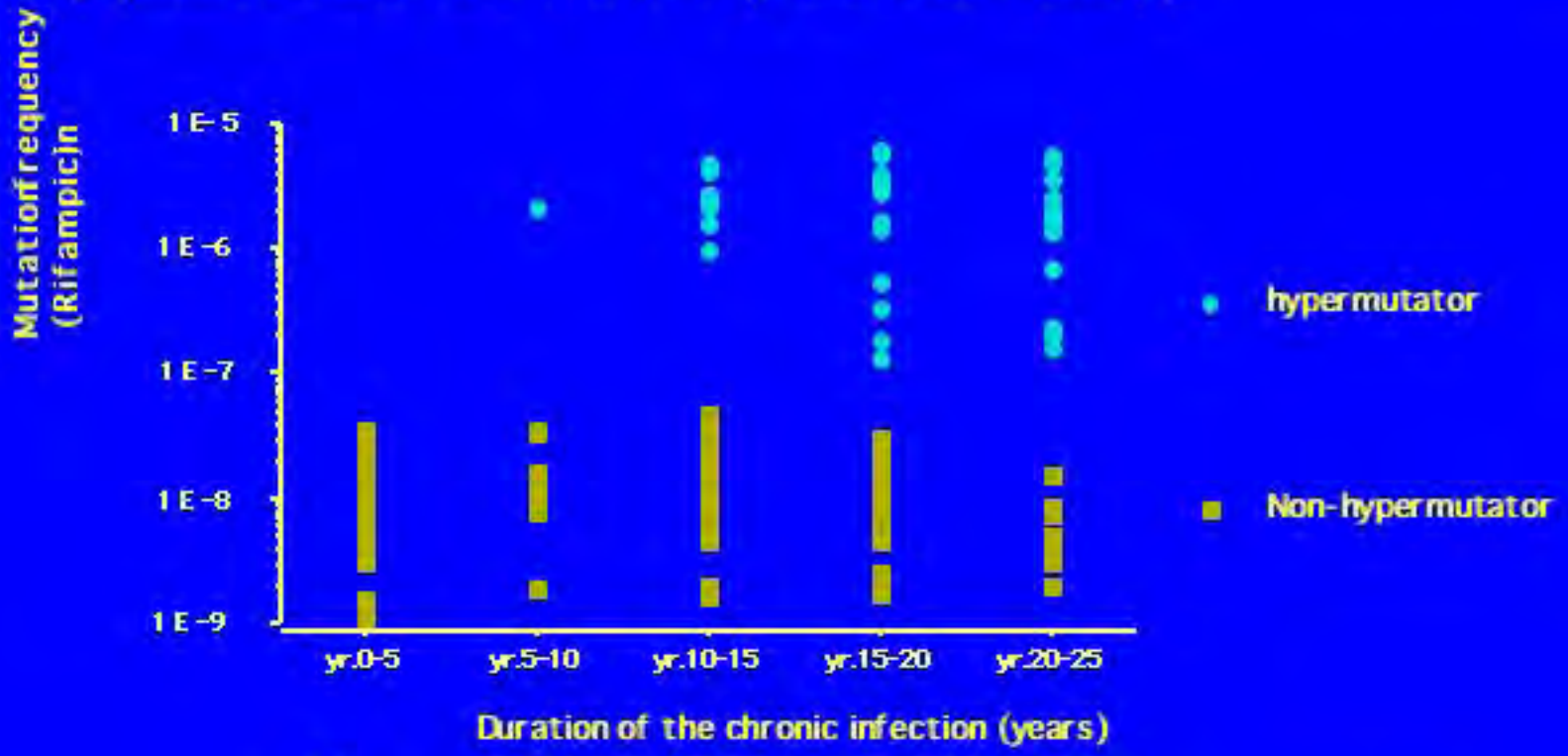
Mutators are more resistant to antibiotics than non-mutators



(Oliver, Science 2000;288:1251)

black: Hp CF, grey: nhpCF, white: non-CF

The frequency of ROS-induced P. aeruginosa mutators increases as time goes by...
Leading to multiply resistant strains selected by antibiotic therapy

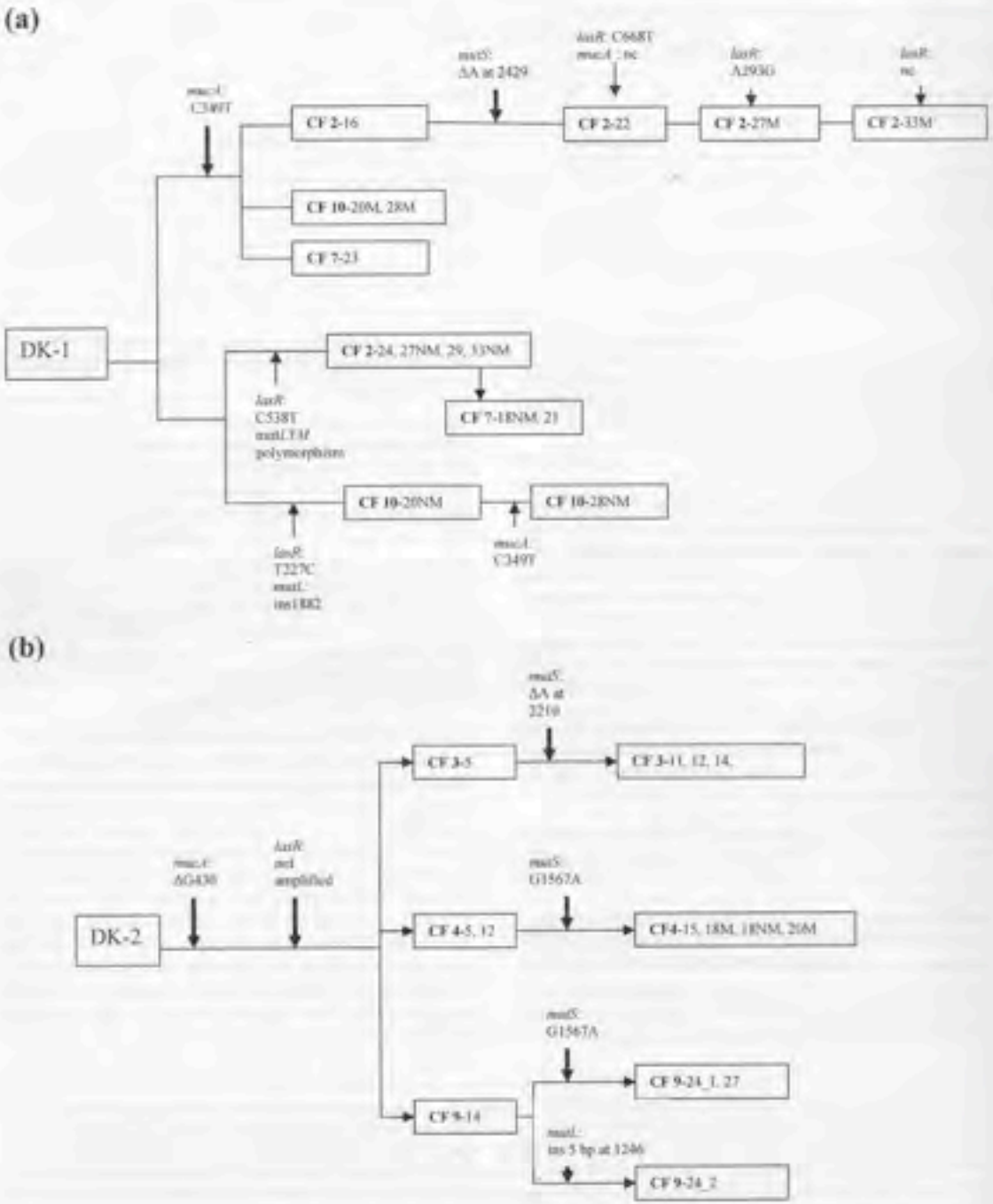


0% 7% 29% 32% 65%

of "random" P. aeruginosa isolates from Danish CF patients

(Ciofu, O., Riis, B., Pressler, T., Poulsen, H.E., Høiby, N.: Occurrence of hypermutable P. aeruginosa in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. Antimicrobial Agents Chemotherapy 49:2276-2282; 2005)

HØIBY 2005



Strong mutators: 20 fold higher than PAO1: 2 x 10^{-8}

Ciofu et al. Genetic adaptation of P. aeruginosa during chronic lung infection of patients with cystic fibrosis: strong and weak mutators with heterogenous genetic backgrounds emerge in *mucA* and/or *lasR* mutants. Microbiology 156:1108-1119; 2010.

mucA	alginate
lasR	quorum sensing

mutS (MMR)	strong mutator
mutL (MMR)	strong mutator
(MMR)	
mutT (GO)	weak mutator
mutY (GO)	weak mutator
mutM (GO)	weak mutator

(MMR: Methyl-directed Mismatch-Repair system)
(GO: 8-oxo-dG – prevents or repair of oxygen lesions)

MOLECULAR MECHANISMS OF FLOUROQUINOLONE RESISTANCE IN P. AERUGINOSA FROM DANISH CF PATIENTS

(Jalal, Ciofu, Høiby, Wretlind. AAC 44:710;2000)

STRAIN	CIPRO-MIC '94 – '97	MUTATION GyrA '94 – '97	MUTATIONS NflxB '94 – '97	MUTATIONS OprN '94 – '97	MUTATIONS O PRj '94 – '97
CF166	1 - 4	- +	+ +	- -	- -
CF222	2 - 8	+ +	+ +	- +	+ +
CF86	2 - 8	+ +	+ +	- +	- +
CF59	2 - 4	+ +	+ +	- -	- -
CF21	0.5 - 4	- +	- +	- -	- -
CF89	0.5 - 2	- +	+ +	- -	- -

Biofilm (B) – Planktonic (P) antimicrobial resistance mechanisms

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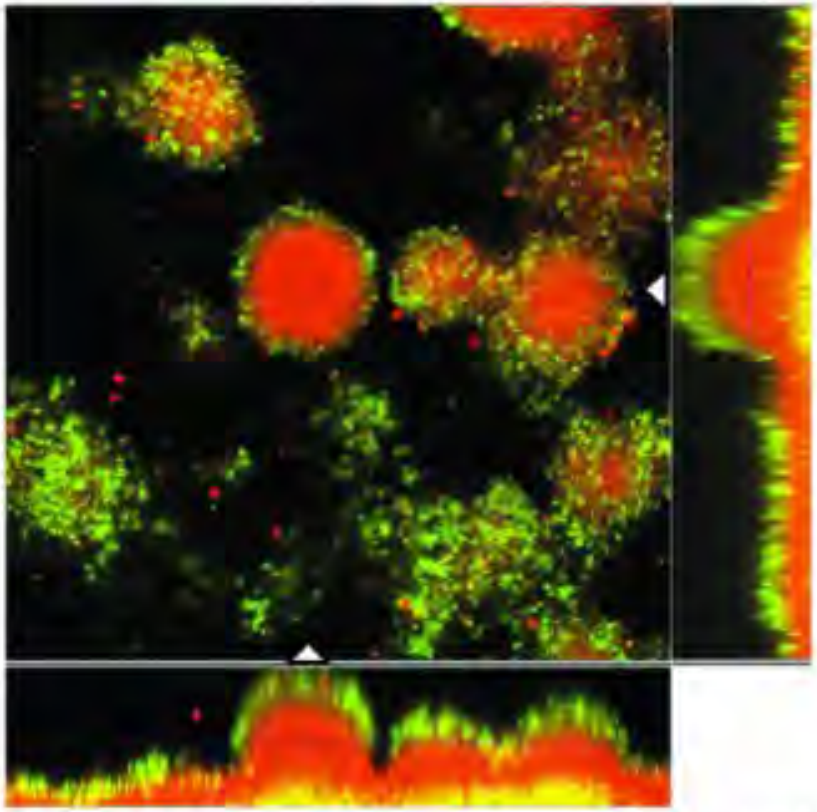
(Høiby, N., Bjarnsholt,T., Givskov, M., Molin, S., Ciofu, O.: Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents 35:322-32; 2010)

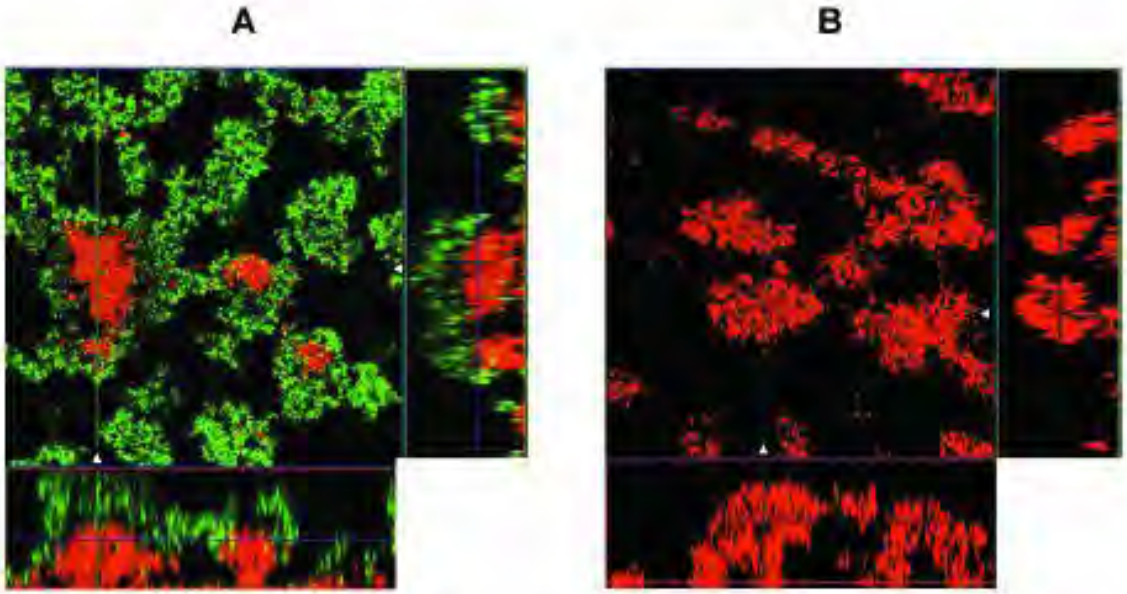
Expression of AmpC β-lactamase in PAO1 biofilm (weak inducer 125 X MIC)



PAO1 with P*ampC-gfp* (ASV): 6 days old biofilm exposed to 100 µg/ml ceftazidime for 4 h. All cells stained red with SYTO 62. Detection level of the monitor: 10 µg/ml ceftazidime. Only the surface layer (green) is induced, Ceftazidime is cleaved and the deeper layer protected.

(Bagge, Antimicrob Agents Chemother. 2004;48: 1168–74)

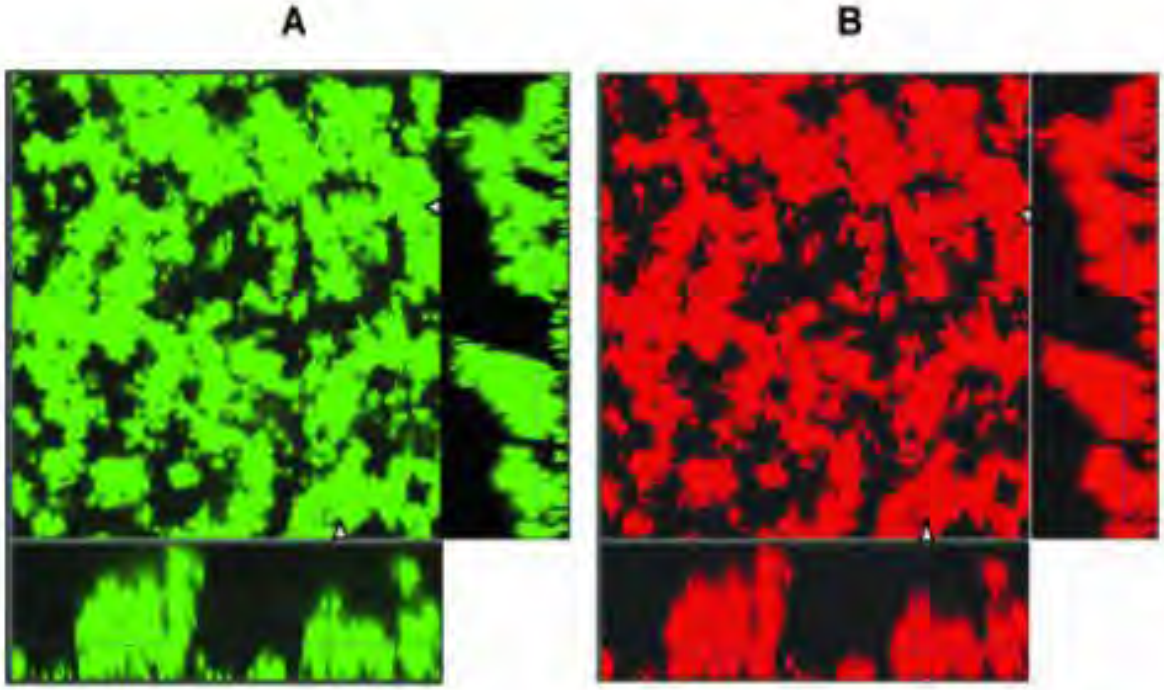
Expression of AmpC β-lactamase in PAO1 biofilm (strong inducer 1/8 MIC)



PAO1 with P*ampC-gfp* (ASV): 6 days old biofilm exposed to 0.5 µg/ml imipenem (1/8 MIC) for 4 hours (A) and uninduced biofilm (B). All cells stained red with SYTO 62 (untreated control). Only the surface layer (green) is induced (Detection level of the monitor ≥0.1 µg/ml imipenem)

(Bagge, Antimicrob Agents Chemother. 2004;48: 1168–74)

Expression of AmpC β-lactamase in PAO1 biofilm (strong inducer 2.5 X MIC)

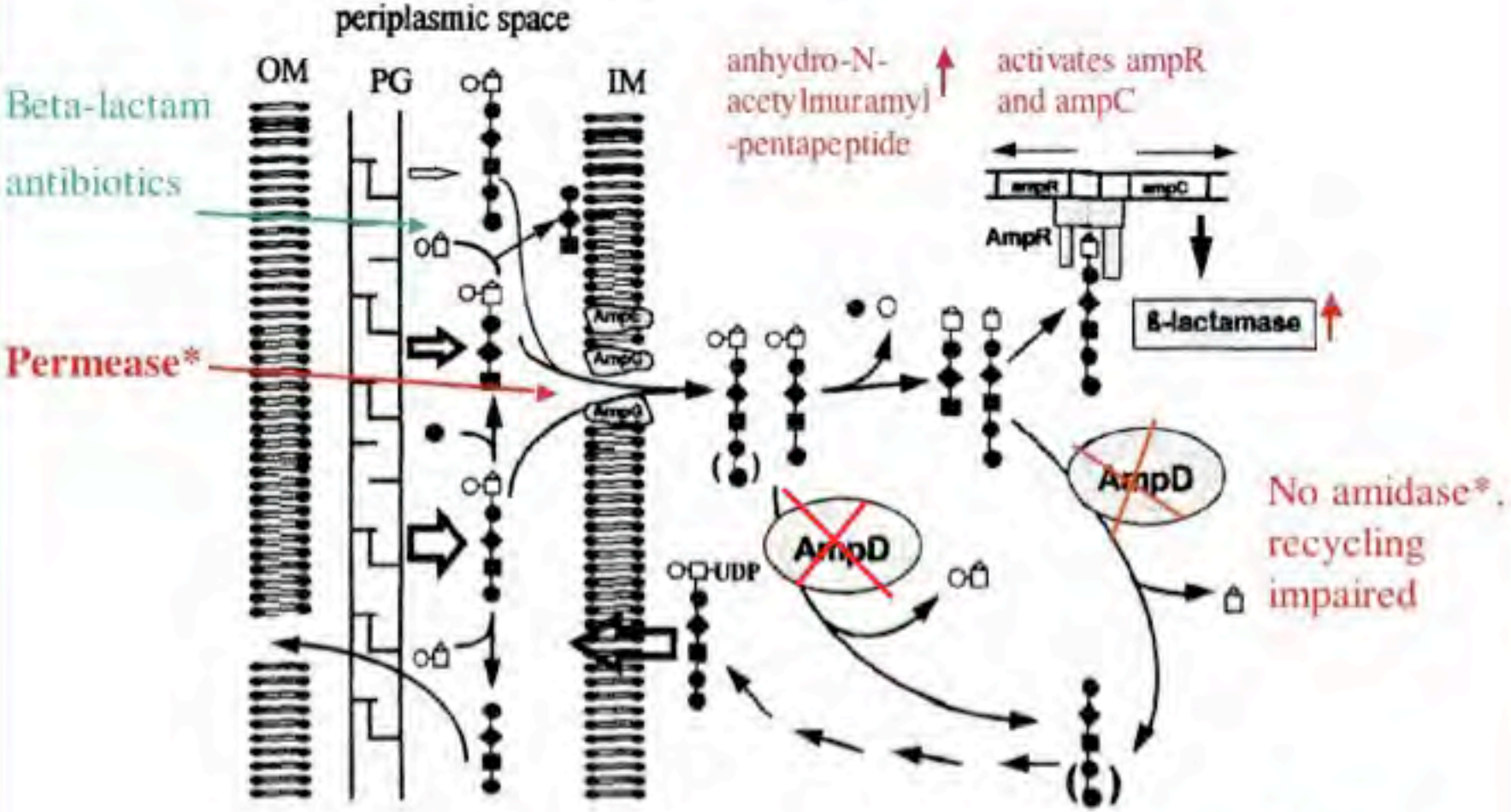


PAO1 with P*ampC-gfp* (ASV): 6 days old biofilm exposed to 10 µg/ml imipenem (2.5x MIC) for 4 hours (A) and uninduced biofilm (B). All cells stained red with SYTO 62(untreated control). All cells are induced (green) (Detection level of the monitor ≥0.1 µg/ml imipenem)

(Bagge, Antimicrob Agents Chemother. 2004;48: 1168–74)

The role of stable derepressed β-lactamase in biofilms

Recycling of peptidoglycan muropeptides and the transcriptional regulation of AmpC β-lactamase production

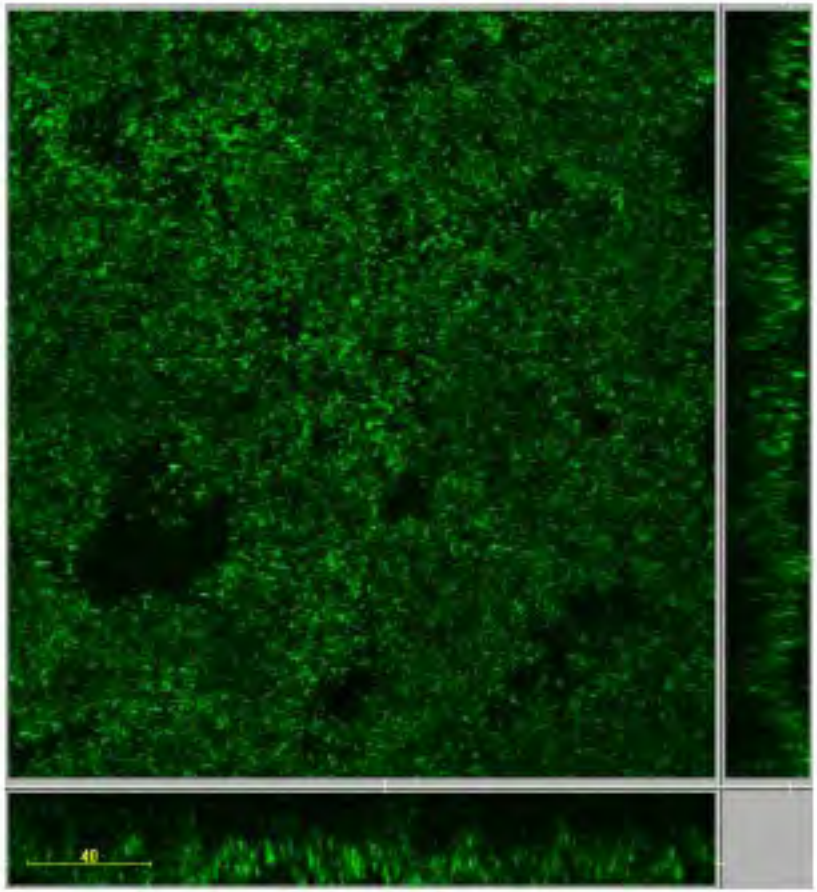


(Dietz, 1997)

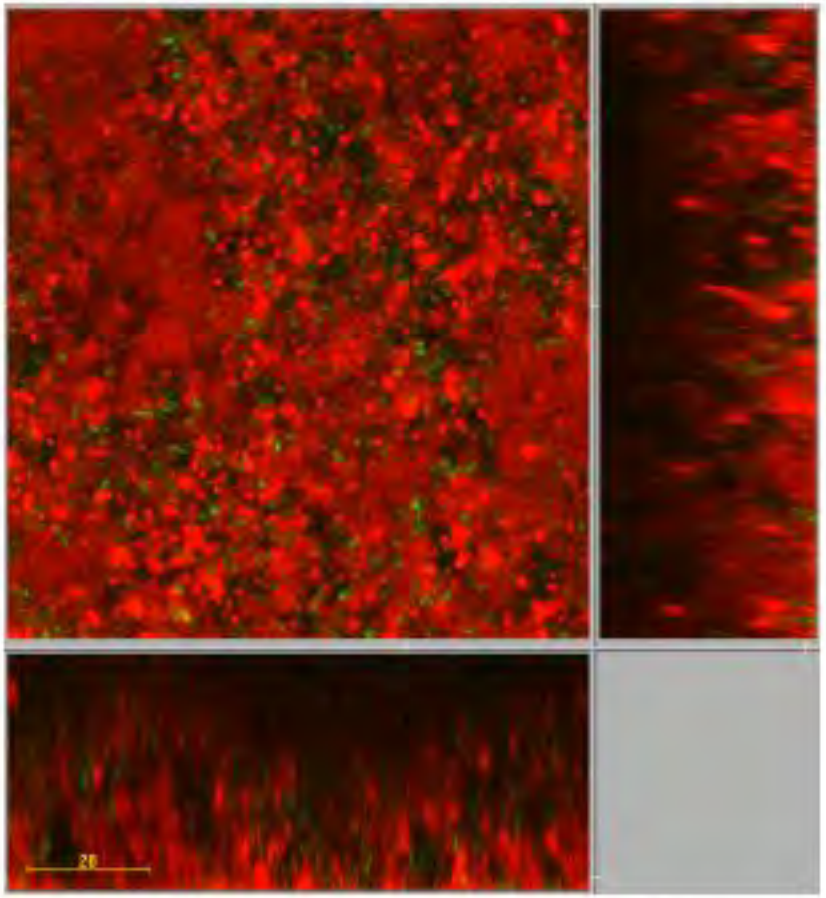
The influence of β-lactamase levels for the treatment of biofilms

	Basal levels	Induced levels
P.a. *ampD*$^{-}$	1050 mU	4255 mU
P.a. *ampD*$^{+}$	3 mU	175 mU

P.a. *ampD*$^{+}$ gfp (low level beta-lactamase) 7 days old biofilm treated with 10 X MIC ceftazidime for 24h, stained with propium iodide (green=slightly induced, red=dead)



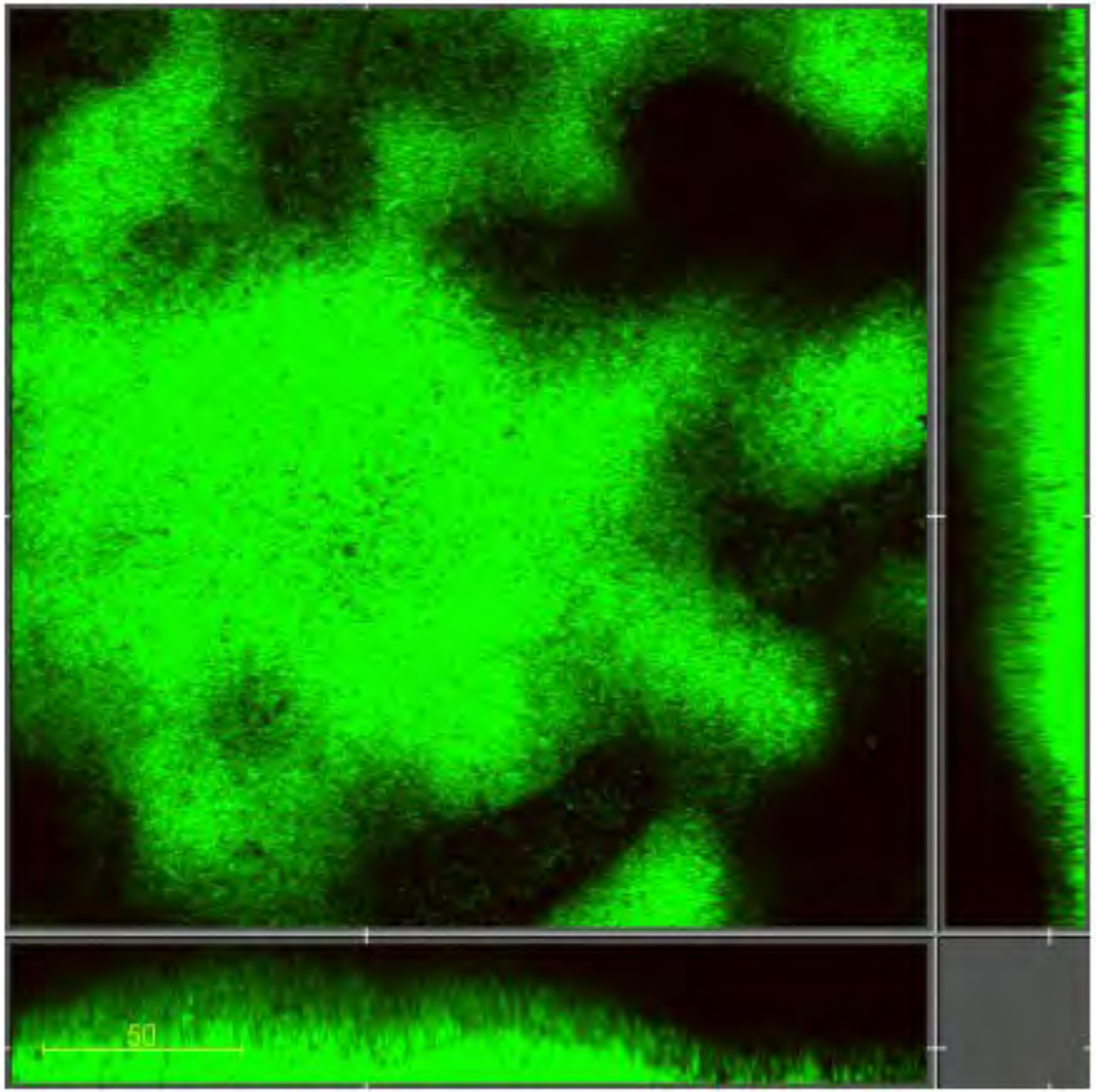
Untreated control biofilm



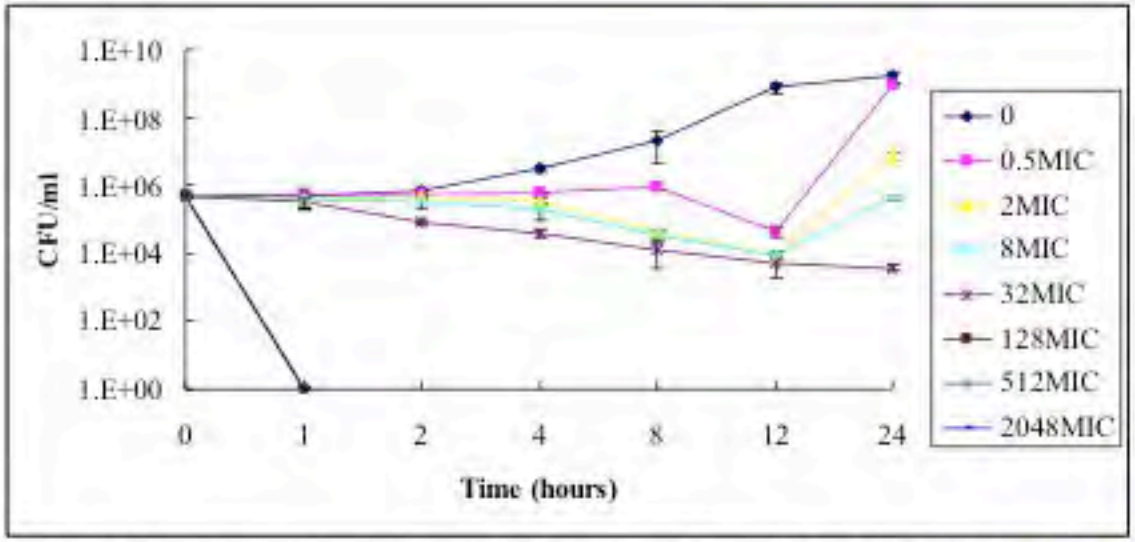
Ceftazidime treated biofilm

(Ciofu, 2005)

P.a. *ampD*$^{-}$ gfp (high level beta-lactamase) 7 days old biofilm treated with 10 X MIC ceftazidime (week inducer) for 24h (induced=green), stained with propium iodide (red = dead)

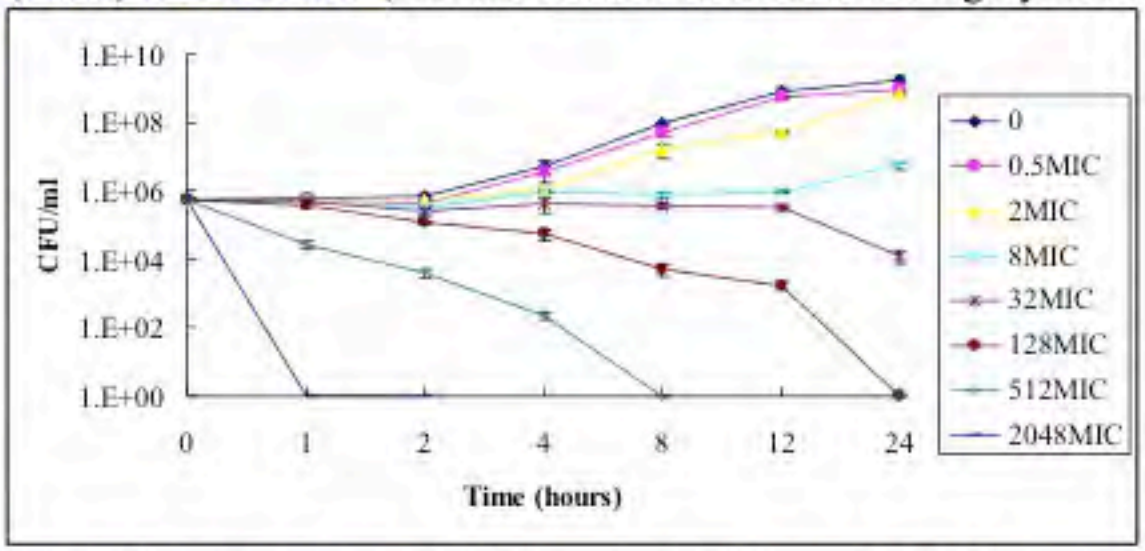


(Ciofu, O., 2005)



Wang, Ciofu, Høiby 2011

Ceftazidime: Time-dependent killing of PAO1 biofilm . The beta-lactamase (basal) levels:12mU (biofilm formed on modified Calgary device)



Ceftazidime: Concentration dependent killing of a PAO1 mutant hyperproducing beta-lactamase. The beta-lactamase (basal) levels:10,000mU

Biofilm (B) – Planktonic (P) antimicrobial resistance mechanisms

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(Høiby, N., Bjarnsholt,T., Givskov, M., Molin, S., Ciofu, O.: Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents 35:322-32; 2010)

Changed biofilm structure due to imipenem exposure of non-mucoid 5 days old PAO1 biofilm

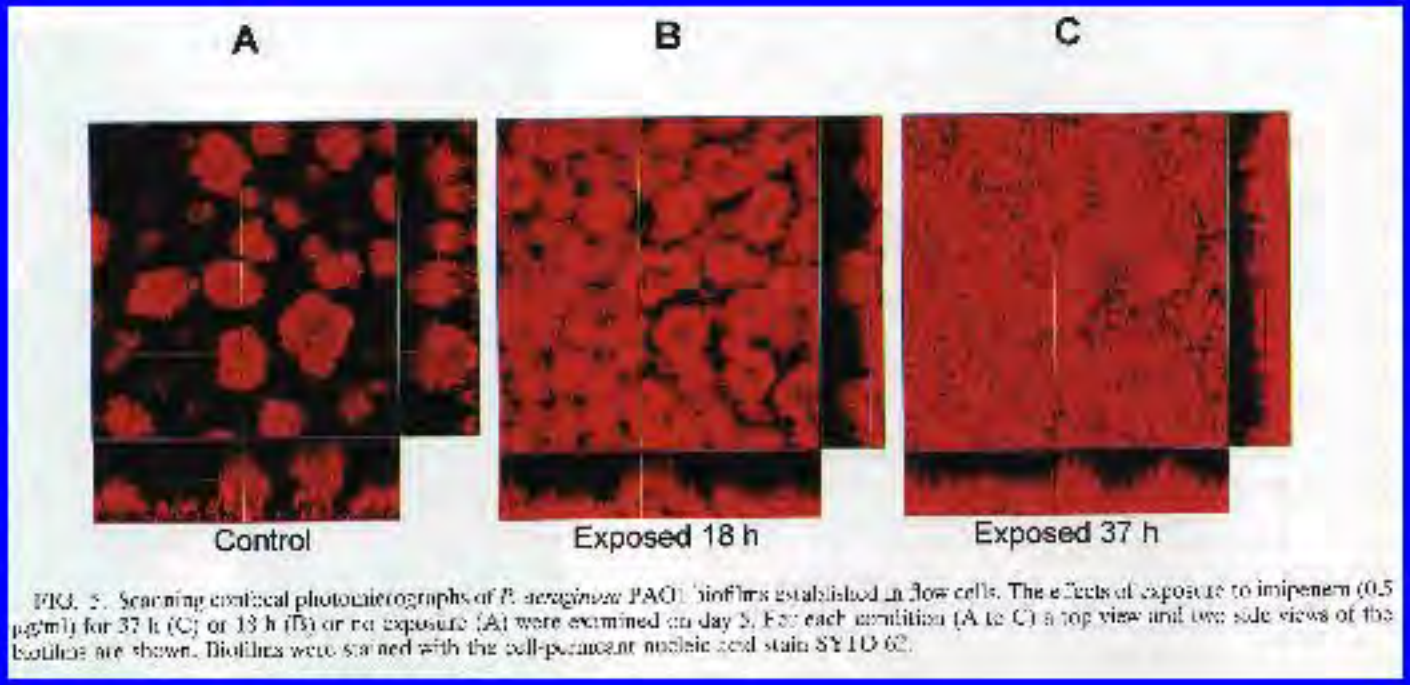


FIG. 5. Scanning confocal photomicrographs of *P. aeruginosa* PAO1 biofilms established in flow cells. The effects of exposure to imipenem (0.5 μg/ml) for 37 h (C) or 18 h (B) or no exposure (A) were examined on day 5. For each condition (A to C) a top view and two side views of the biofilms are shown. Biofilms were stained with the cell-permeant nucleic acid stain SYTO 62.

(Bagge, Schuster, Hentzer, Ciofu, Givskov, Greenberg & Høiby: Pseudomonas aeruginosa biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. AAC 48:1175-87; 2004)

HØIBY 2004

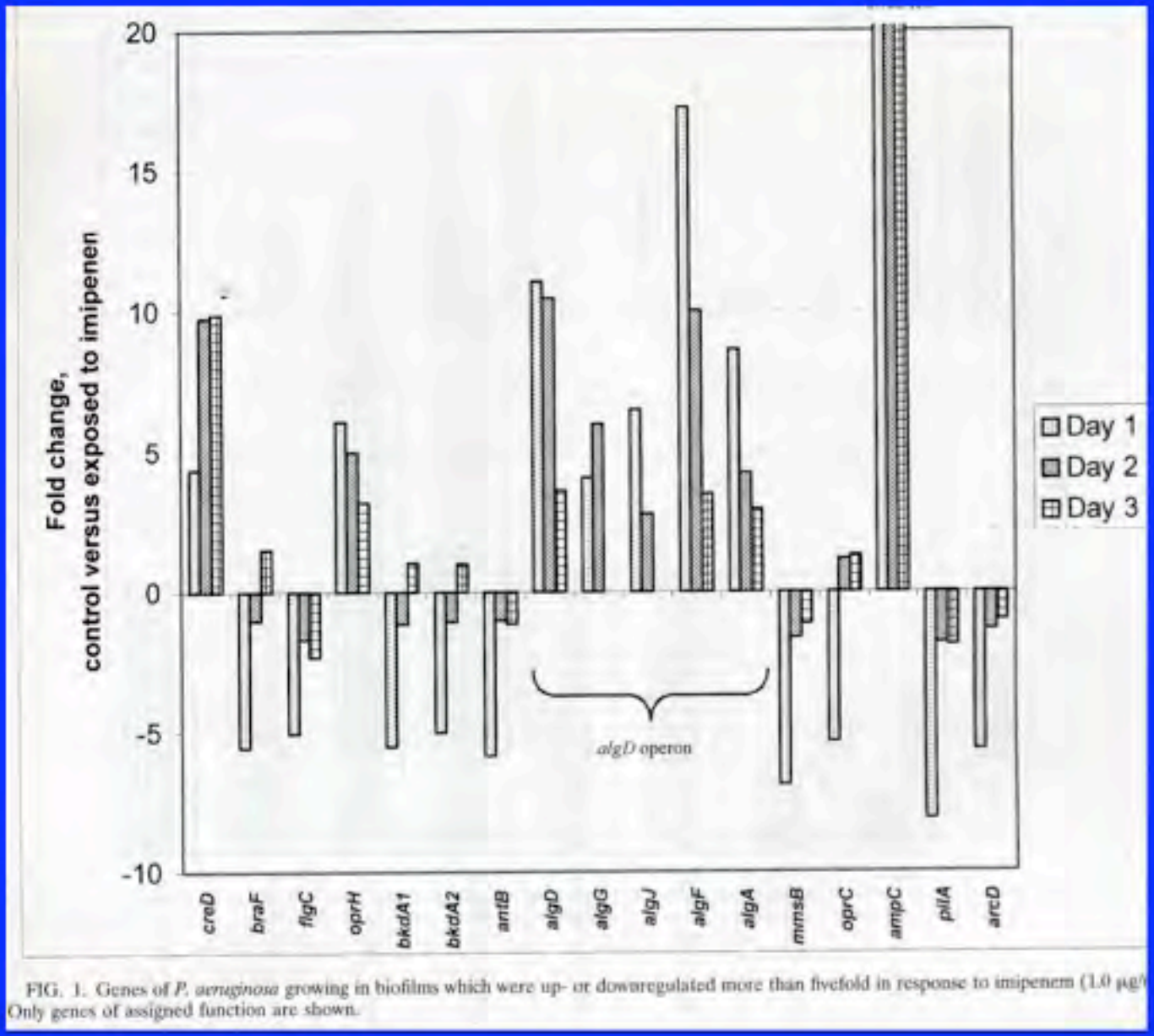


FIG. 1. Genes of *P. aeruginosa* growing in biofilms which were up- or downregulated more than fivefold in response to imipenem (1.0 μg/
Only genes of assigned function are shown.

(Bagge, Schuster, Hentzer, Ciofu, Givskov, Greenberg & Høiby: Pseudomonas aeruginosa biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. AAC 48:1175-87; 2004)

HØIBY 2004

Effect of induction in P. aeruginosa PAO1 5 days old biofilm with 0.5 μg/ml imipenem for 18 or 37 hr. Staining of alginate with ConA-FITC

PAO1 non-induced control biofilm

PAO1 induced with imipenem for 18 h

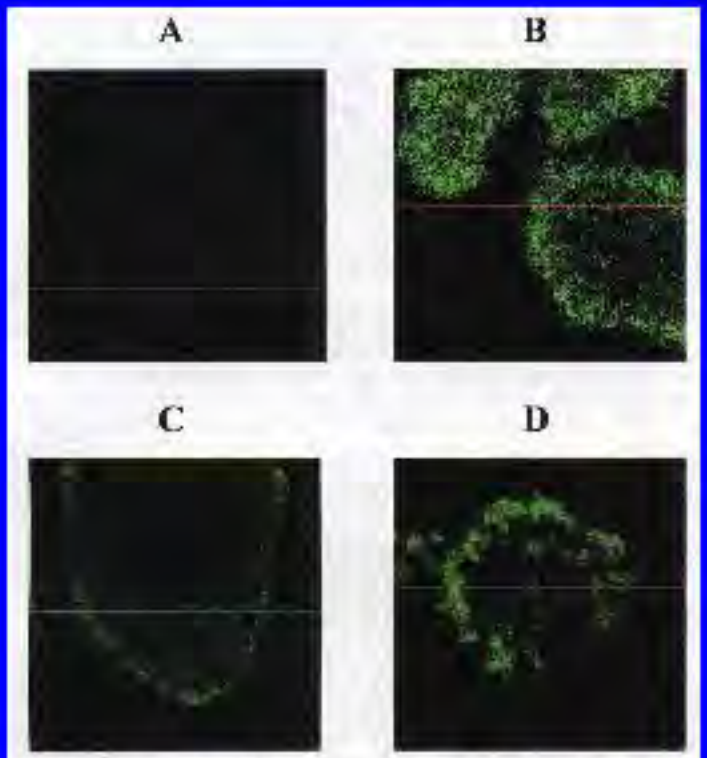


FIG. 6. Staining confocal photomicrographs of *P. aeruginosa* biofilms established in flow cells. Select microcolonies stained with ConA-FITC, which reveals polysaccharides such as alginate, are shown from the top. (A) PAO1 biofilm not exposed to antibiotics; (B) PDO300 (a derivate of PAO1 that constitutively overproduces alginate) not exposed to antibiotics; (C) PAO1 exposed to imipenem for 18 h; (D) PAO1 exposed to imipenem for 37 h.

Mucoid PDO300 non-induced PAO1 control biofilm

PAO1 induced with imipenem for 37 h

(Bagge, Schuster, Hentzer, Ciofu, Givskov, Greenberg & Høiby: Pseudomonas aeruginosa biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. AAC 48:1175-87; 2004)

HØIBY 2004

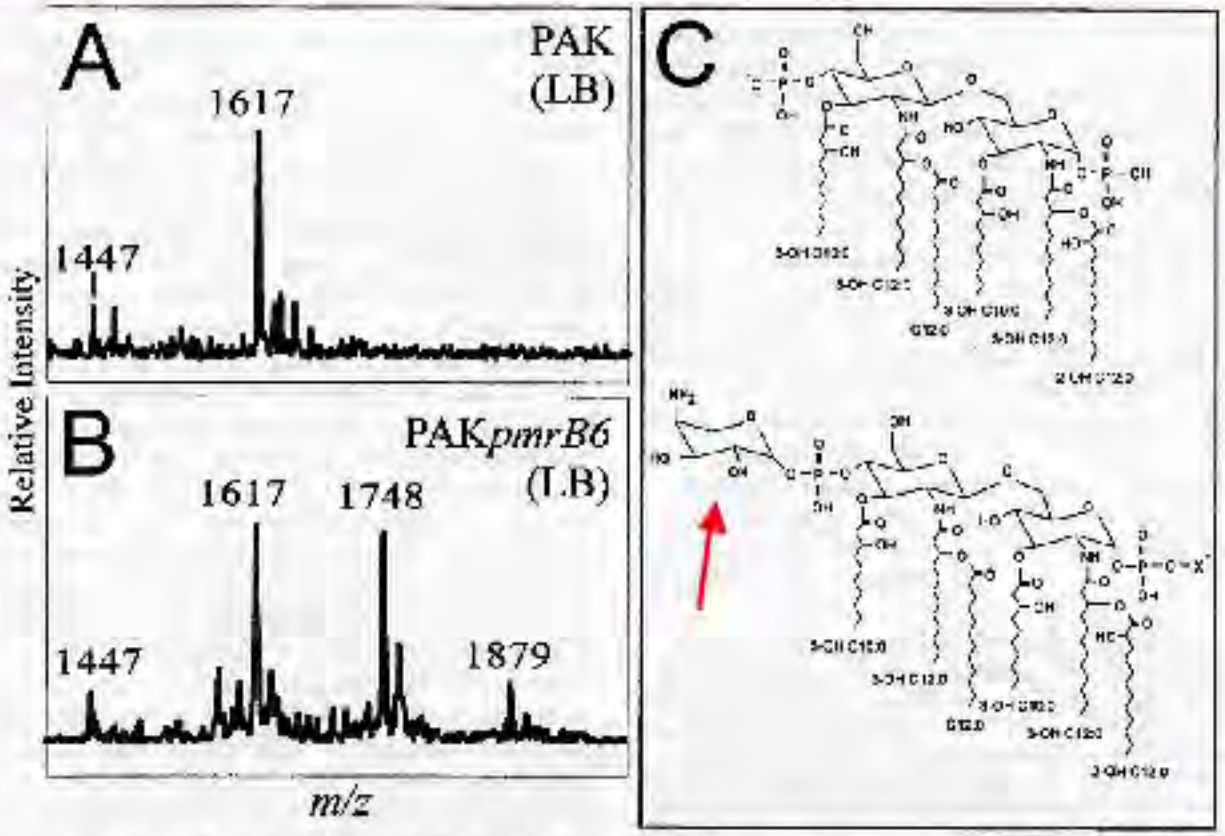


FIG. 2. Association of *pmrB* mutations with constitutive addition of aminoarabinose to *P. aeruginosa* lipid A. (A) MALDI-TOF negative ion mode analysis of lipid A purified from *P. aeruginosa* strain PAK. In the negative-ion mode, observed molecular species lack at least one proton [M − H]$^-$. The difference between [M—H]$^-$ at *m/z* 1,447 and *m/z* 1,617 ($\Delta m/z$ = 170) in the mass spectrum indicates the loss of 3-hydroxydecanoate from position 3 of the reducing diglucosamine (right-hand ring in panel C). (B) Mass spectrum for lipid A from strain PAK*pmrB6*. The difference between [M—H]$^-$ at *m/z* 1,617, 1,748, and 1,879 ($\Delta m/z$ = 131) indicates the addition of aminoarabinose to the 1 and 4′ phosphates of lipid A. (C) Structure of *P. aeruginosa* lipid A without and with aminoarabinose. The X^- symbol at the right side of the lower structure represents either H (corresponding to *m/z* 1,748) or aminoarabinose (corresponding to *m/z* 1,879). The fatty acids depicted are 3-hydroxydecanoate (3-OH $C_{10:0}$), laurate ($C_{12:0}$), 2-hydroxylaurate (2-OH $C_{12:0}$), and 3-hydroxylaurate (3-OH $C_{12:0}$).

Moskowitz et L.: PmrAB, a two-component regulatory system of P. aeruginosa that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. J. Bact. 186:575-79; 2004.

HØIBY 2005

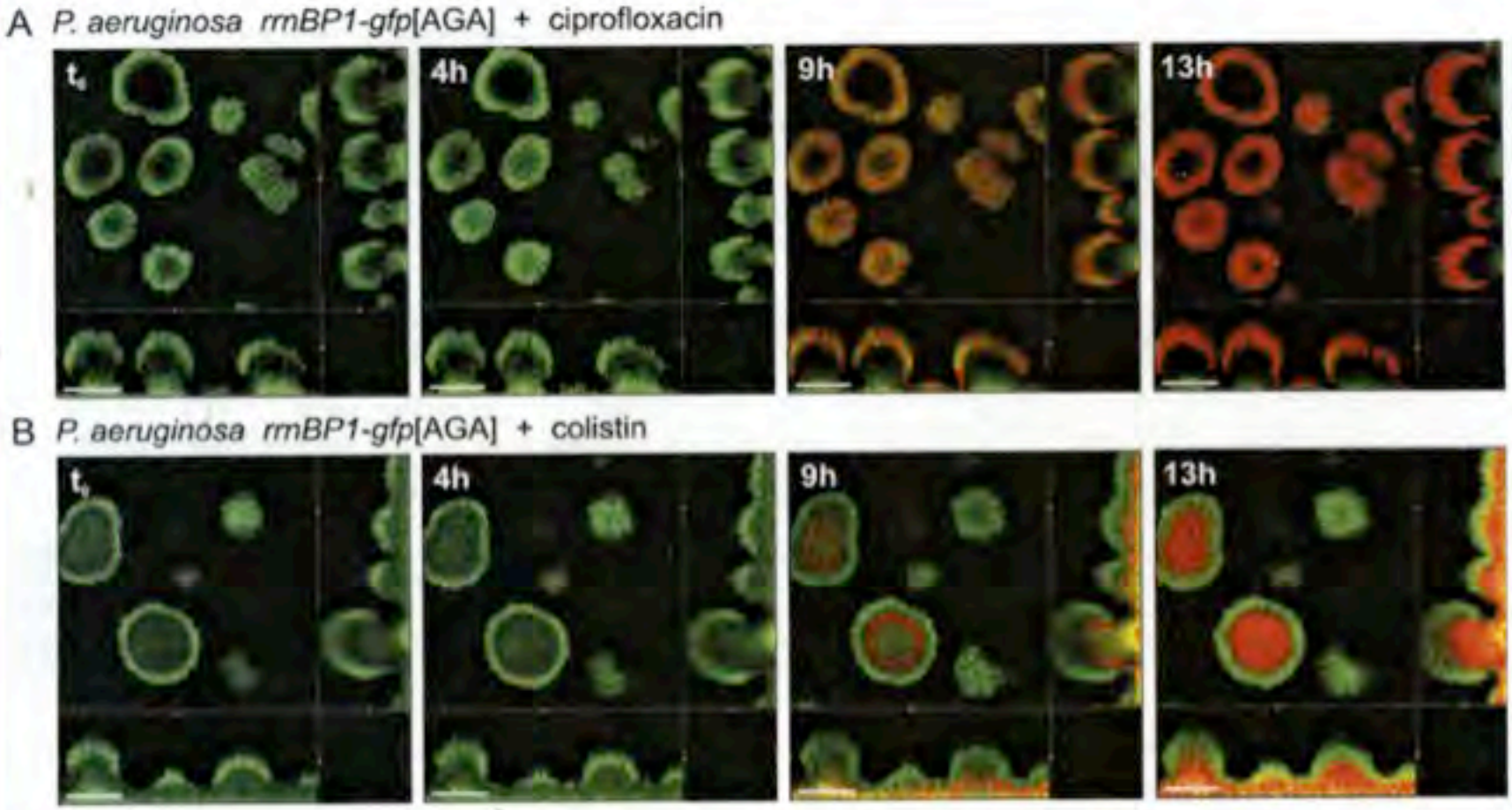


Fig. 3. Targeting distinct metabolic/physiological subpopulations in *P. aeruginosa* biofilms by ciprofloxacin and colistin. Biofilms of *P. aeruginosa* PAO1 Tn7-*rmnB*P1–*gfp*[AGA] were grown for 4 days and then continuously exposed to either 60 μg ml^{-1} ciprofloxacin and propidium iodide (A), or to 25 μg ml^{-1} colistin and propidium iodide (B). Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and 4, 9 and 13 h subsequent to the beginning of treatment. The images show a horizontal section with two flanking images representing sections in the xz and yz planes respectively. Metabolic active cells appear green because of expression of Gfp[AGA] under control of the ribosomal promoter *rmnB*P1 and dead cells appear red, as a result of staining with the dead cell indicator propidium iodide.

Pamp, Gjermansen, Krogh Johansen, Tolker-Nielsen et al. Tolerance to the antimicrobial peptide colistin in P. aeruginosa biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. Molecular Microbiology 68:223-40; 2008

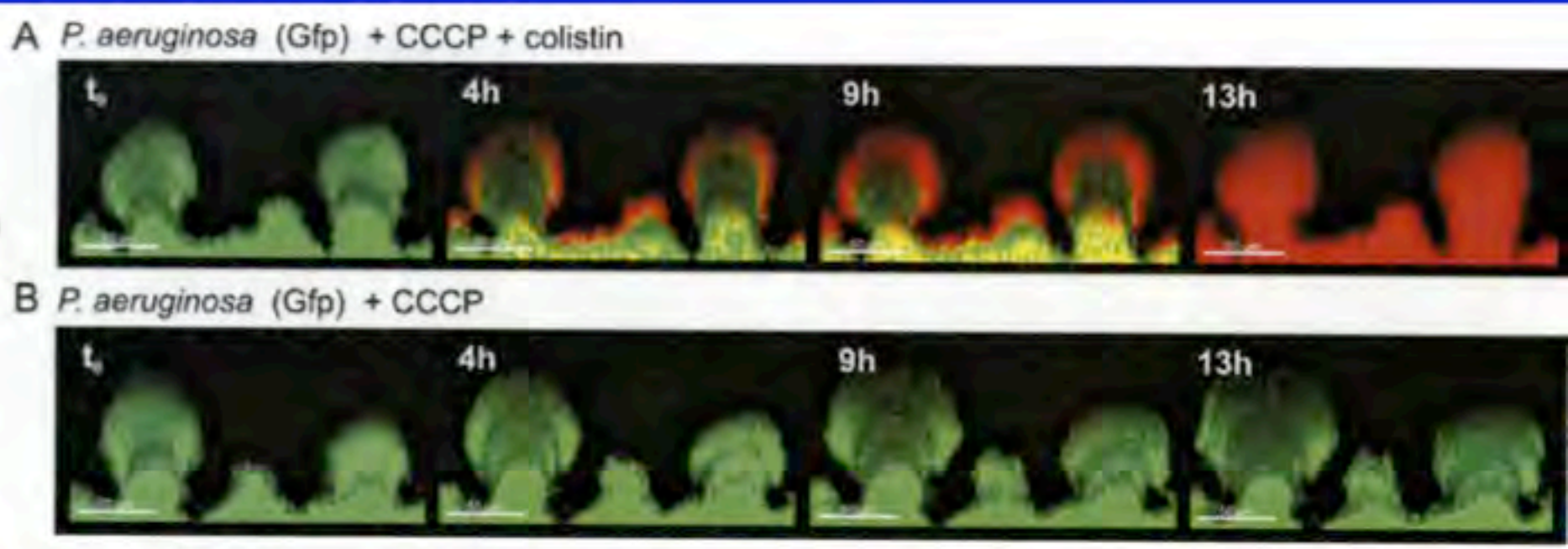
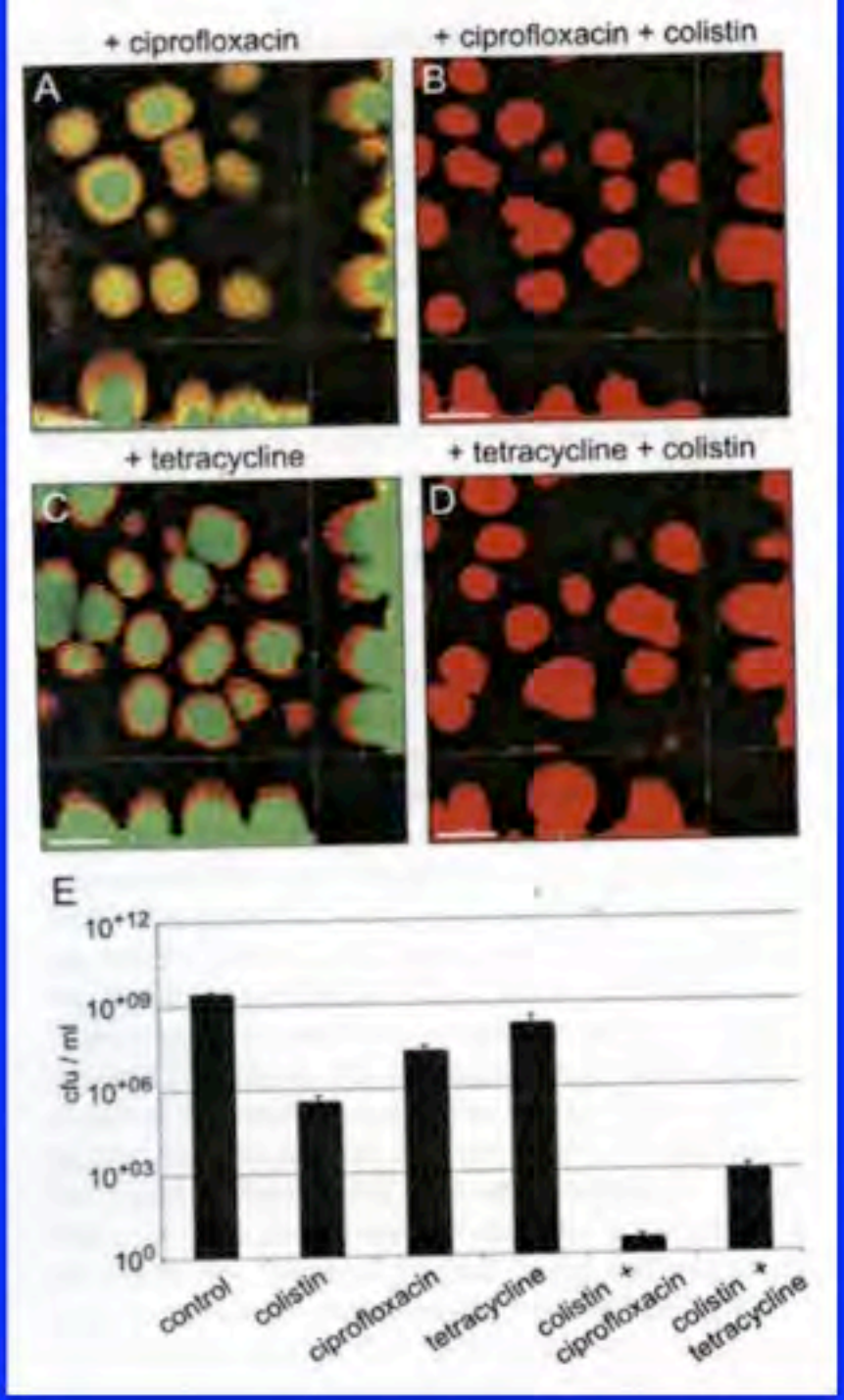


Fig. 4. Biofilm cells exposed to CCCP become sensitive to colistin. Biofilms of *P. aeruginosa* PAO1 Gfp were grown for 4 days and then continuously exposed to either 30 μM CCCP, 25 μg ml^{-1} colistin and propidium iodide (A), or, as control, to 30 μg ml^{-1} CCCP and propidium iodide (B). Confocal laser scanning micrographs were acquired at time point t_0 (prior to exposure) and 4, 9 and 13 h subsequent to the beginning of treatment. The images represent vertical sections of biofilms respectively. Live cells appear green because of expression of Gfp and dead cells appear red, because of staining with the dead cell indicator propidium iodide.

Pamp, Gjermansen, Krogh Johansen, Tolker-Nielsen et al. Tolerance to the antimicrobial peptide colistin in P. aeruginosa biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. Molecular Microbiology 68:223-40; 2008



Pamp, Gjermansen, Krogh Johansen, Tolker-Nielsen et al. Tolerance to the antimicrobial peptide colistin in P. aeruginosa biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. Molecular Microbiology 68:223-40; 2008

Synergism – or additive effect – of:
colistin and ciprofloxacin
and of
colistin and tetracycline

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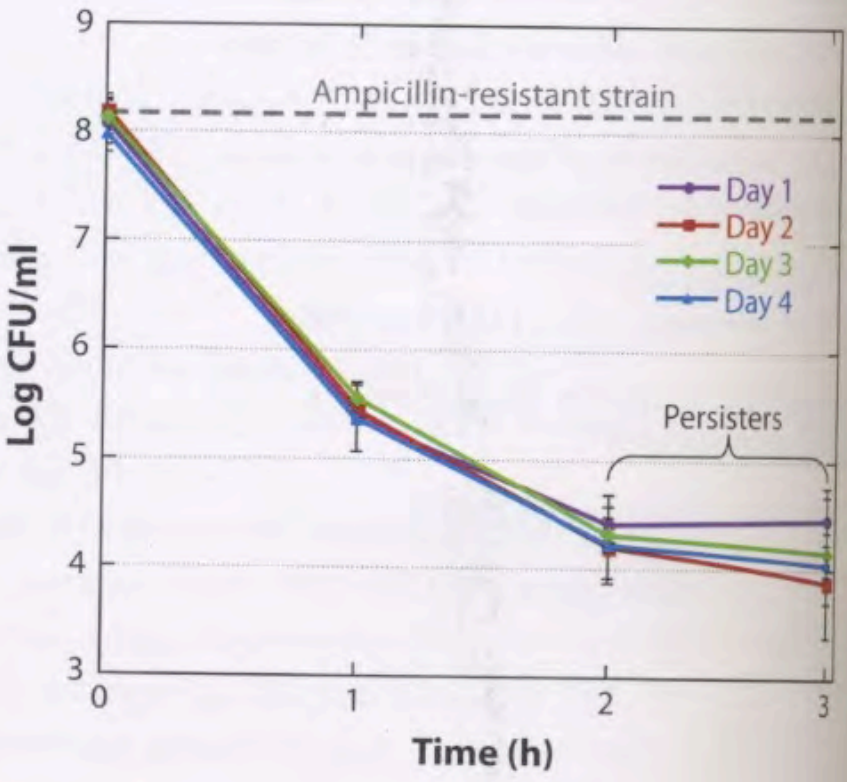

Figure 1

A test for persister heritability. An exponentially growing culture of *Escherichia coli* was treated with a high dose of ampicillin at time 0. After lysis, surviving persisters were reinoculated in fresh medium, cultured, and ampicillin was applied again. The dashed line indicates how an ampicillin-resistant strain would have behaved. Based on Reference 46.

K. Lewis. Persister cells. Ann. Rev. Microbiol. 64:357-72; 2010.

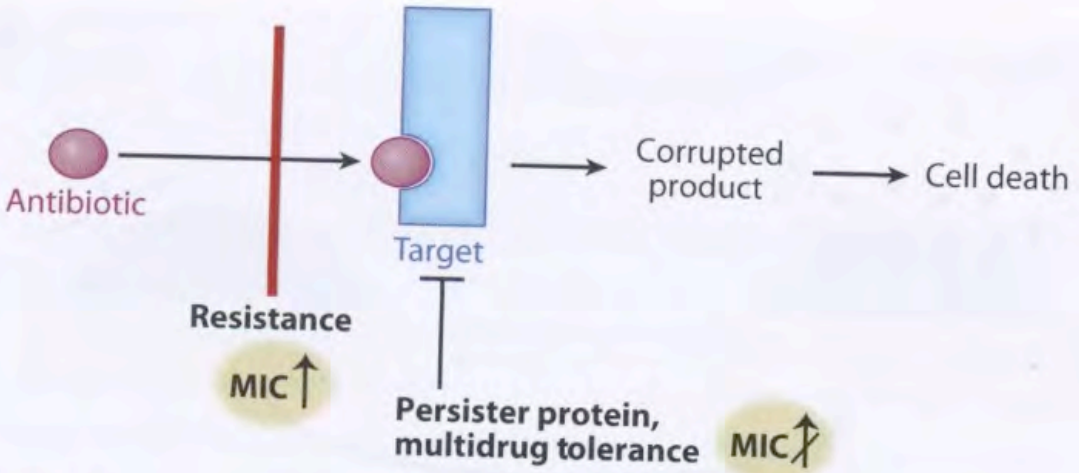

Figure 2

Resistance versus tolerance. Bactericidal antibiotics kill cells by forcing the active target to produce corrupted products. Streptomycin interrupts translation, producing toxic misfolded peptides (21). Inhibition of peptidoglycan synthesis by β-lactams causes induction of autolysins and cell death by a process that is still poorly understood (9); fluoroquinolones bind to the DNA gyrase and convert it to DNA endonuclease (41). A recent study reported that bactericidal antibiotics also lead to the production of reactive oxygen species, contributing to cell death (48). Persister proteins act by blocking the target, so no corrupted product can be produced. By contrast, all resistance mechanisms prevent the antibiotic from binding to the target.

K. Lewis. Persister cells. Ann. Rev. Microbiol. 64:357-72; 2010.

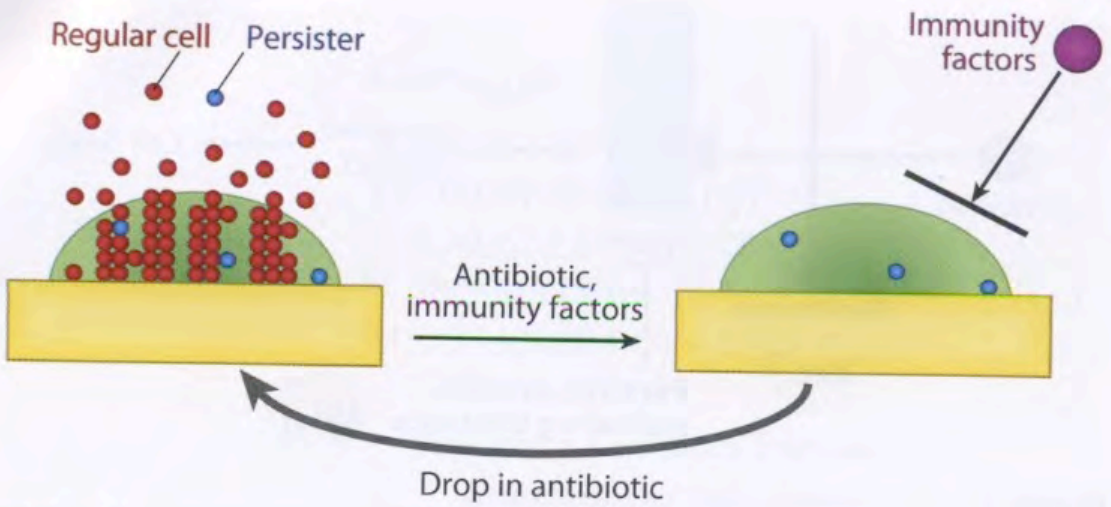

Figure 3

A model of a relapsing biofilm infections. Regular cells and persister cells form in the biofilm and are shed off into surrounding tissue and the bloodstream. Antibiotics kills regular cells, and the immune system eliminates escaping persister cells. The matrix protects persister cells from the immune system, and when the concentration of the antibiotic drops, they repopulate the biofilm, causing a relapse.

K. Lewis. Persister cells. Ann. Rev. Microbiol. 64:357-72; 2010.

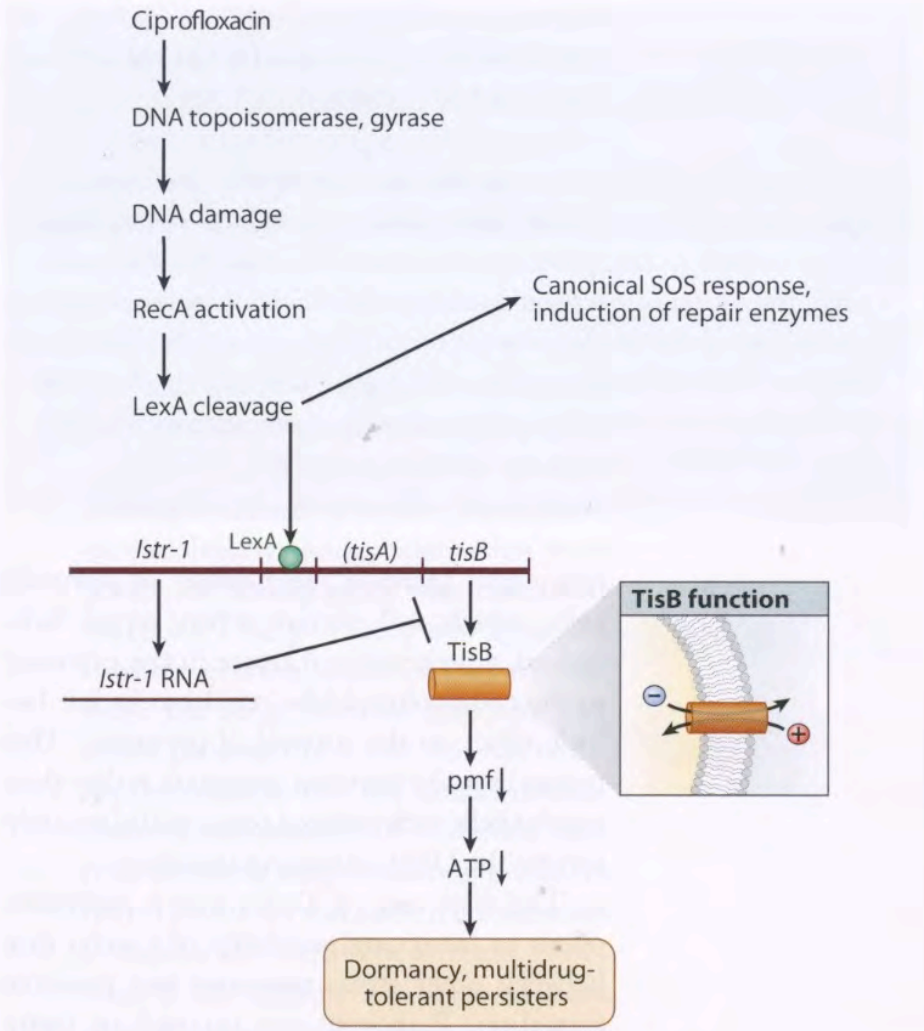

Figure 6

A model of TisB-dependent persister formation in *Escherichia coli*. A fluoroquinolone antibiotic causes DNA damage by converting the DNA gyrase and topoisomerase to endonucleases. This activates the RecA protein, which in turns activates the LexA repressor, causing it to cleave. The canonical SOS response is induced, and repair enzymes that contain *lex* boxes in their promoter regions are transcribed. The Lex repressor also controls the expression of the TisB toxin, a small cationic membrane-acting agent. Decrease in the proton motive force (pmf) and ATP shuts down target functions, including DNA topoisomerase and gyrase, and a dormant persister is formed.

K. Lewis. Persister cells. Ann. Rev. Microbiol. 64:357-72; 2010.

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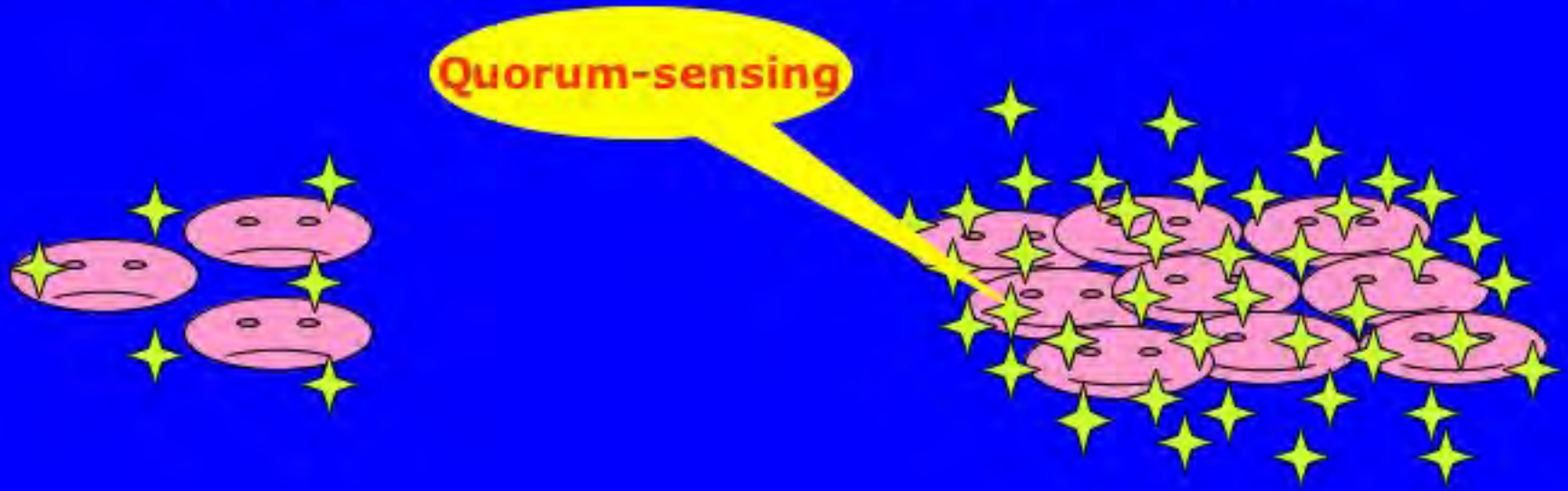
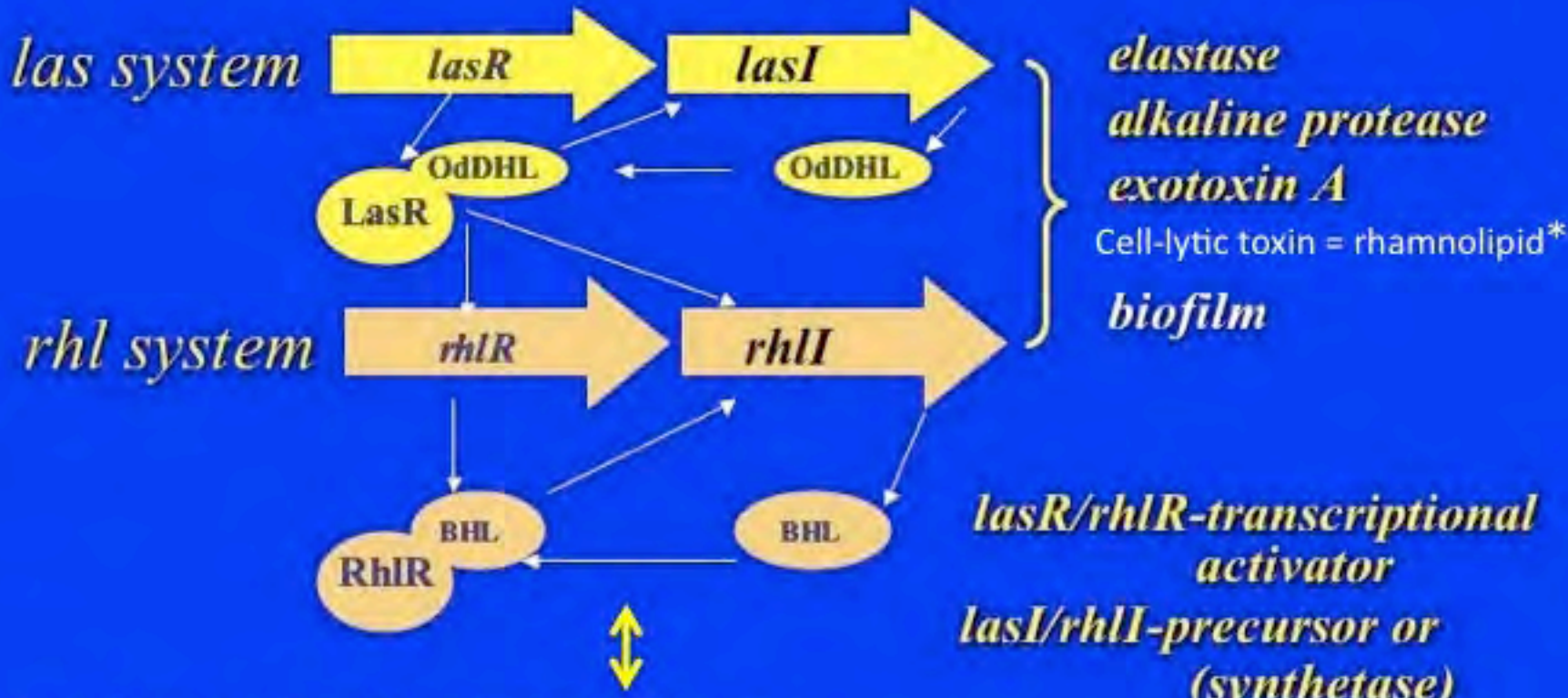
(Høiby, N., Bjarnsholt,T., Givskov, M., Molin, S., Ciofu, O.: Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents 35:322-32; 2010)

Cell-density depending Quorum sensing: Bacteria communicate by means of Acyl-Homoserine-Lactons - also in biofilms



P. aeruginosa AHLs

CELL DENSITY DEPENDENT QUORUM SENSING SIGNALING IN PSEUDOMONAS AERUGINOSA - IMPORTANT FOR BIOFILM STRUCTURE - AND RESISTANCE!



*Jensen, P.Ø. Et al.: Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by Pseudomonas aeruginosa. Microbiology 153:1329-38; 2007.

Hong Wu 2004

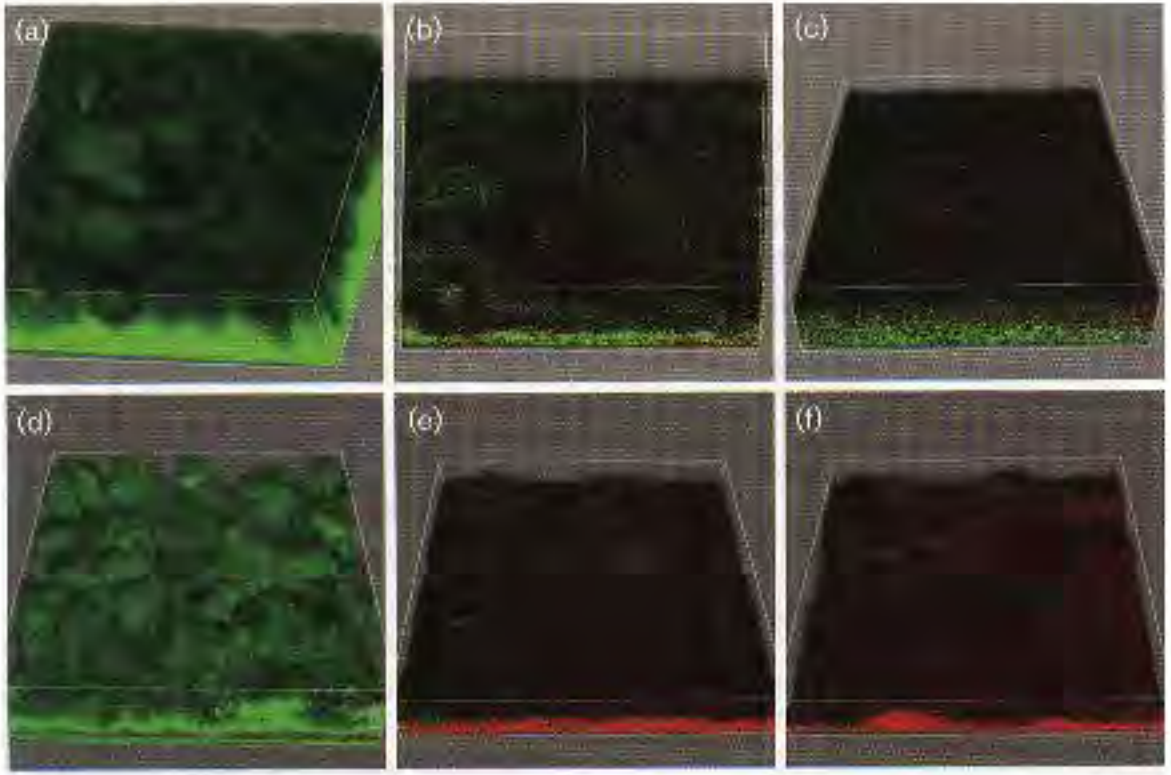
Control Tobramycin 10μg/ml 20 μg/ml

Wild-type PAO1

(+QS)

ΔlasR ΔrhlR mutant PAO1

(no QS)



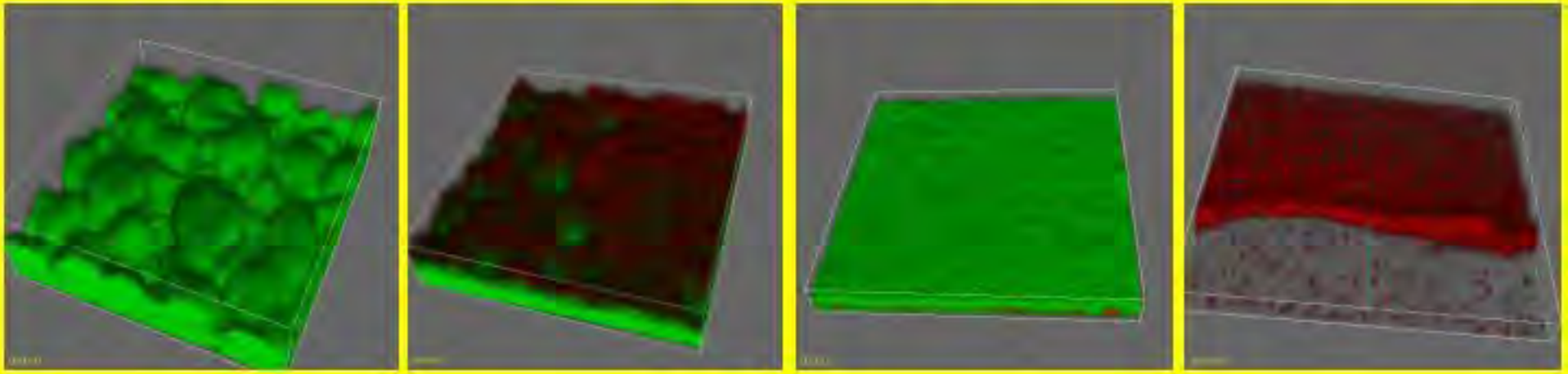
Tobramycin treatment of non-mucoid P. aeruginosa biofilm. To 3-days old biofilm was added tobramycin (b, e: 10 μg/ml, c, f: 20 μg/ml. A, c controls. Red: dead P. aeruginosa cells, Green: alive P. aeruginosa cells. Photo after 48h tobramycin treatment, Propidium iodide staining (live-dead)

(Bjarnsholt, Jensen, Burmølle, Hentzer, Haagensen, Hougen, Calum, Madsen, Moser, Molin, Høiby, Givskov. Pseudomonas aeruginosa tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. Microbiology 151:373-83; 2005)

HØIBY 2005

QSI by Garlic

PAO1 | 24h tobramycin | Garlic | Tobra + Garlic



-3 days old *P. aeruginosa* *_lasRrhlR* biofilms were grown +/- Garlic extract

-The appropriate biofilms were exposed to **tobramycin** for 24 h

-Biofilm viability assayed using **LIVE/DEAD** ***Bac*Light**
Green Read

(Bjarnsholt et al: Garlic blocks quorum sensing and promotes rapid clearing of pulmonary Pseudomonas aeruginosa infections. Microbiology 151:3873-3880;2005)

Hoffmann et al. Effects of Azithromycin on P. aeruginosa (AAC 51:3677-87; 2007)

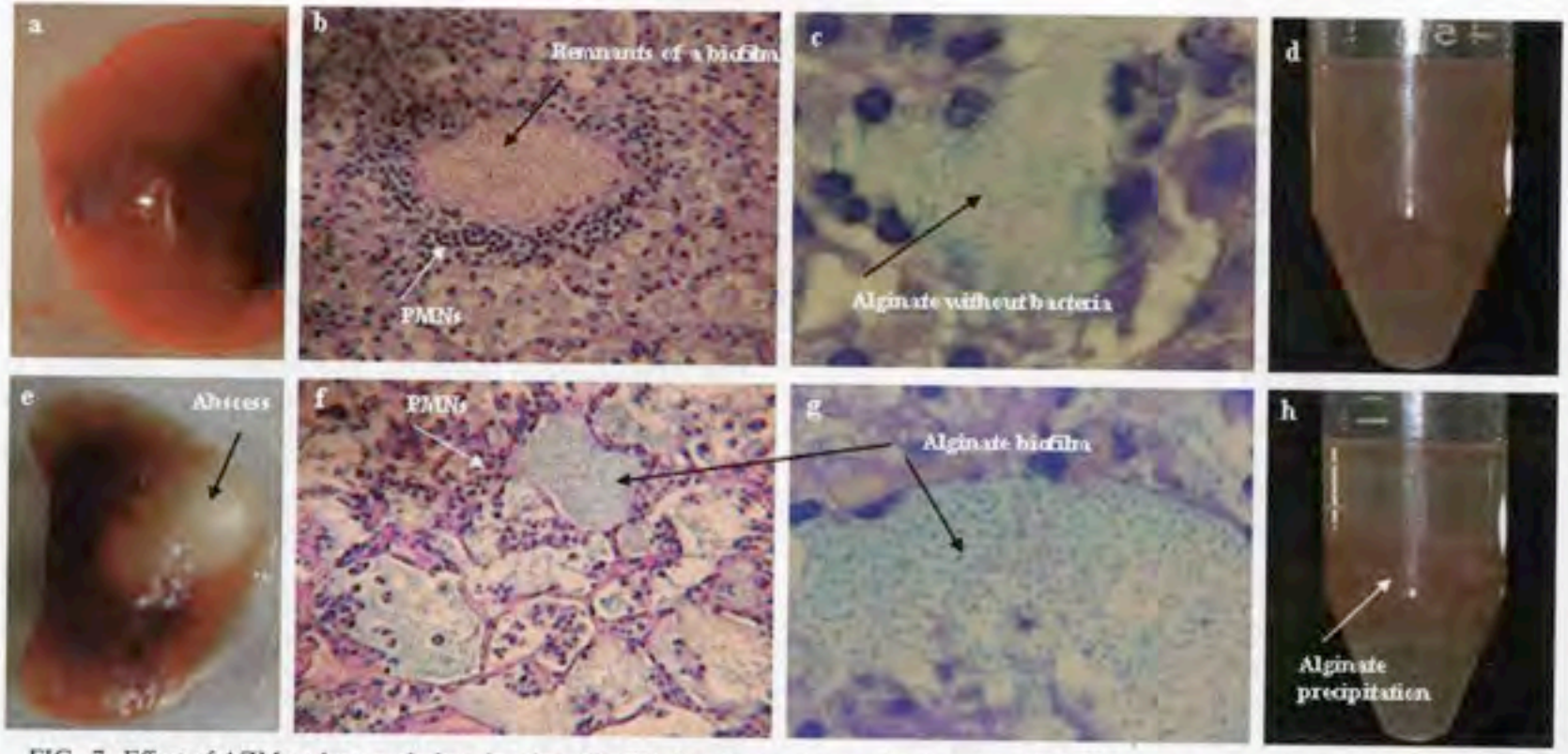


FIG. 7. Effect of AZM on lung pathology in mice with CF after *P. aeruginosa* infection. (a to d) AZM-treated mouse indicating mild pathology with no abscess (a) and probably remnants of a biofilm (black arrow) with a pronounced PMN infiltration (white arrow) (magnification, ×40) (b), and residues of alginate (blue color) without bacteria could be found (magnification, ×100) (c). HE and Alcian blue staining were used. (d) Lung homogenate without alginate precipitation. (e to h) Saline-treated mouse showing a big lung abscess (black arrows) (e) and large *P. aeruginosa* biofilms (black arrows) in the alveoli (f and g) encapsulated in alginate (blue color) surrounded by PMNs (white arrow). Magnifications, ×40 (f) and ×100 (g). HE and Alcian blue staining was used. (h) Alcohol precipitation of alginate from mouse lung homogenate (white arrow).

Biofilm (B) – Planktonic (P) antimicrobial resistance mechanisms

- Stationary phase physiology, low oxygen – slow growth (B)
- Mutations – hypermutators (B, P)
- Beta-lactamase, penetration barrier, alginate, binding to matrix (B, P)
- Tolerance, adaptive resistance, efflux pumps (B, P)
- Persisters (B, P)
- High cell density – Quorum sensing (B, P)

(Høiby, N., Bjarnsholt,T., Givskov, M., Molin, S., Ciofu, O.: Antibiotic resistance of bacterial biofilms. International Journal of Antimicrobial Agents 35:322-32; 2010)

Hit Hard: Antibiotic therapy against P. aeruginosa in cystic fibrosis

Intermittent P. aeruginosa colonization – early, aggressive eradication therapy:

-Oral ciprofloxacin + nebulised colistin (or tobramycin) for 3 weeks to 3 months (in case of failure)

Chronic P. aeruginosa infection – maintenance therapy = chronic suppressive therapy for the rest of the patients life:

•Daily nebulised colistin/tobramycin (aztreonam, ciprofloxacin) and daily azithromycin (QSI and antiinflammatory) and i.v. therapy for 2 weeks every 3 months:

•Tobramycin + piperacillin/tazobactam
- or + ceftazidime
- or + aztreonam
- or + imipenem
- or + meropenem
- and sometimes oral ciprofloxacin
- and + nebulised colistin or tobramycin